Molecular Microbiology (2007) **64**(6), 1466–1485 doi:10.1111/j.1365-2958.2007.05734.x

Type VI secretion is a major virulence determinant in *Burkholderia mallei*

Mark A. Schell,[1] Ricky L. Ulrich,[2] Wilson J. Ribot,[2] Ernst E. Brueggemann,[3] Harry B. Hines,[3] Dan Chen,[4] Lyla Lipscomb,[1] H. Stanley Kim,[5] Jan Mrázek,[1] William C. Nierman[4] and David DeShazer[2]*

[1]*Department of Microbiology, University of Georgia, Athens, GA 30602, USA.*

[2]*Bacteriology Division and* [3]*Integrated Toxicology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702-5011, USA.*

[4]*The Institute for Genomic Research, Rockville, MD 20850, USA.*

[5]*Korea University Medical College, Seoul, Korea.*

Summary

Burkholderia mallei **is a host-adapted pathogen and a category B biothreat agent. Although the** ***B. mallei*** **VirAG two-component regulatory system is required for virulence in hamsters, the virulence genes it regulates are unknown. Here we show with expression profiling that overexpression of** ***virAG*** **resulted in transcriptional activation of ~60 genes, including some involved in capsule production, actin-based intracellular motility, and type VI secretion (T6S). The 15 genes encoding the major sugar component of the homopolymeric capsule were up-expressed >2.5-fold, but capsule was still produced in the absence of** ***virAG*****. Actin tail formation required** ***virAG*** **as well as** ***bimB*****,** ***bimC*** **and** ***bimE*****, three previously uncharacterized genes that were activated four- to 15-fold when VirAG was overproduced. Surprisingly, actin polymerization was found to be dispensable for virulence in hamsters. In contrast, genes encoding a T6S system were up-expressed as much as 30-fold and mutations in this T6S gene cluster resulted in strains that were avirulent in hamsters. SDS-PAGE and mass spectrometry demonstrated that BMAA0742 was secreted by the T6S system when** ***virAG*** **was overexpressed. Purified His-tagged BMAA0742 was recognized by glanders antiserum from a horse, a human and mice, indicating that this Hcp-family protein is produced** ***in vivo*** **during infection.**

Accepted 6 April, 2007. *For correspondence. E-mail david.deshazer@amedd.army.mil; Tel. (+1) 301 619 4871; Fax (+1) 301 619 8351.

Introduction

Glanders is a disease caused by *Burkholderia mallei* and it is one of the oldest infectious diseases known (McFadyean, 1904; Waag and DeShazer, 2004). *B. mallei* is an obligate animal pathogen whose natural hosts are horses, donkeys and mules, but infections can also occur in felines, camels and goats. Humans are accidental hosts of *B. mallei* and the majority of cases have been the result of occupational contact with infected horses. Whereas equines are generally infected orally, the primary route of infection in humans is contamination of skin abrasions or mucus membranes with nasal discharge or skin lesion exudate from an infected animal. *B. mallei* is highly infectious in a laboratory setting, especially via the aerosol route (Stewart, 1904; Hunter, 1936; Howe and Miller, 1947; Srinivasan *et al*., 2001; Kohler, 2006). Diagnosis and treatment can be challenging and no licensed vaccines are currently available. As it is widely believed that *B. mallei* has the potential for use as a biological weapon, it has been designated as a category B select agent by the CDC (Lehavi *et al*., 2002; Rotz *et al*., 2002; Voskuhl *et al*., 2003).

Virulence in *B. mallei* is multifactorial and several virulence determinants have been identified and characterized using animal models of infection, including capsular polysaccharide and type III secretion (DeShazer *et al*., 2001; Ulrich and DeShazer, 2004; Ribot and Ulrich, 2006). In addition, a complex quorum-sensing network and a two-component transcriptional regulatory system (VirAG) are required for maximal virulence in hamsters, but the genes controlled by these regulators are unknown (Nierman *et al*., 2004; Ulrich *et al*., 2004). *B. mallei* is a facultative intracellular pathogen that can invade, survive and replicate in epithelial and phagocytic cell lines (Harley *et al*., 1998; Ribot and Ulrich, 2006). This pathogen escapes the phagocytic vacuole into the cytoplasm where it can use actin-based motility for intra- and intercellular spread (Ribot and Ulrich, 2006; Stevens *et al*., 2005a), perhaps to avoid detection by the host humoral immune response (Carlsson and Brown, 2006). An autotransported protein responsible for *Burkholderia* intracellular motility has been identified and designated BimA (Stevens *et al*., 2005a,b), but the role of actin tail formation as a virulence determinant has not been examined in an animal model of infection.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JUN 2007	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Type VI secretion is a major virulence determinant in Burkholderia mallei. Molecular Microbiology 64:1466-1485	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Schell MA, Ulrich RL, Ribot WJ, Brueggemann EE, Hines HB, Chen D, Lipscomb L, Kim HS, Mrázek J, Nierman WC, Deshazer D	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	**TR-06-138**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

The original document contains color images.

14. ABSTRACT

Burkholderia mallei is a host-adapted pathogen and a category B biothreat agent. Although the B. mallei VirAG two-component regulatory system is required for virulence in hamsters, the virulence genes it regulates are unknown. Here we show with expression profiling that overexpression of virAG resulted in transcriptional activation of approximately 60 genes, including some involved in capsule production, actin-based intracellular motility, and type VI secretion (T6S). The 15 genes encoding the major sugar component of the homopolymeric capsule were up-expressed > 2.5-fold, but capsule was still produced in the absence of virAG. Actin tail formation required virAG as well as bimB, bimC and bimE, three previously uncharacterized genes that were activated four- to 15-fold when VirAG was overproduced. Surprisingly, actin polymerization was found to be dispensable for virulence in hamsters. In contrast, genes encoding a T6S system were up-expressed as much as 30-fold and mutations in this T6S gene cluster resulted in strains that were avirulent in hamsters. SDS-PAGE and mass spectrometry demonstrated that BMAA0742 was secreted by the T6S system when virAG was overexpressed. Purified His-tagged BMAA0742 was recognized by glanders antiserum from a horse, a human and mice, indicating that this Hcp-family protein is produced in vivo during infection.

15. SUBJECT TERMS

Burkholderia mallei, glanders, type VI secretion system, melioidosis, T6S, SDS-PAGE, virulence gene expression

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**20**	

Proteobacteria have evolved at least five distinct secretion pathways, types I–V, to transport proteins from the cytoplasm to the external environment (Desvaux *et al*., 2004). Recently, a new secretion pathway was discovered and termed type VI secretion (T6S) (Dudley *et al*., 2006; Pukatzki *et al*., 2006). This new secretion pathway is conserved in numerous Gram-negative pathogens and symbionts that interact closely with eukaryotic cells (Folkesson *et al*., 2002; Moore *et al*., 2002; Bladergroen *et al*., 2003; Das and Chaudhuri, 2003; Rao *et al*., 2004; Dudley *et al*., 2006; Mougous *et al*., 2006; Pukatzki *et al*., 2006); however, T6S systems (T6SSs) are also found in other presumably 'solitary' bacteria. Little is known about the structure and organization of the T6S apparatus and the proteins secreted by this system apparently do not contain N-terminal signal sequences. In addition, T6S gene clusters are under highly regulated genetic control (Bladergroen *et al*., 2003; Zheng *et al*., 2005; Dudley *et al*., 2006; Mougous *et al*., 2006; Pukatzki *et al*., 2006). Investigation of the role of T6SSs in host–pathogen interactions have been very limited but it is likely that T6SSs will be shown to play an important role in pathogenesis in mammalian hosts (Potvin *et al*., 2003; Nano *et al*., 2004). Most of the T6SSs studied to date appear to secrete a protein with sequence similarity to the haemolysin-coregulated protein (Hcp) of *Vibrio cholerae* and related proteins in many other Gram-negative bacteria (Williams *et al*., 1996). The function of these Hcp-like proteins (COG3517) remains elusive, but the structure of the *Pseudomonas aeruginosa* Hcp1 protein was recently determined (Mougous *et al*., 2006). Hcp1 forms a hexameric ring structure with an interior diameter of ~40 A. It was hypothesized that this T6S protein may form an external channel through which other macromolecules are transported.

In this study we characterized a new T6SS in *B. mallei*, one of four T6SSs encoded in its genome. We found that it was required for virulence in the hamster model of glanders infection and was activated by the VirAG two-component system and BMAA1517, a newly discovered AraC-type regulator. Moreover, we showed that the Hcp-family protein encoded in this cluster was secreted at fairly high levels and was immunogenic in mice, horses and humans, indicating that it is produced *in vivo*. The results presented here demonstrate that T6SSs are likely to be important virulence determinants for other Gram-negative mammalian pathogens.

Results

Overexpression of virAG *activates transcription of gene clusters involved in capsule production, actin tail formation and T6S*

Previously we demonstrated that a mutation in the two-component sensor kinase regulatory gene *virG* (*BMAA0746*) rendered wild-type *B. mallei* ATCC 23344 avirulent in hamsters (Nierman *et al*., 2004). The goal of this investigation was to identify and characterize the virulence genes that are transcriptionally activated by the VirAG two-component system. Several preliminary whole genome expression profiling experiments of wild-type *B. mallei* and its *virG* mutant showed only a few minor differences in gene expression (data not shown), suggesting the unknown environmental cues recognized by the VirAG two-component system were absent under our laboratory growth conditions. In follow-up experiments, *virAG* was overexpressed in wild-type *B. mallei* from a constitutive promoter on a broad-host-range plasmid (pBHR2-*virAG*) and its mid-log phase transcriptome compared with that of *B. mallei* harbouring the pBHR2 vector alone (Table 1). As expected, *virAG* was up-expressed an average of 15-fold. Additionally, four large gene clusters and ~13 individual unlinked genes were up-expressed at least twofold in *B. mallei* harbouring pBHR2-*virAG* (Table 1). The first of these clusters was *wcbF-wcbS* (*BMA2302-BMA2288*). These capsule biosynthesis genes were up-expressed an average of twofold. Interestingly, these genes are involved in biosynthesis of 6-deoxy-β-D-*manno*-heptopyranose, the sugar component of the homopolymeric *B. mallei* capsule (DeShazer *et al*., 2001). In contrast, the adjacent genes (*BMA2304-BMA2309*), likely involved in translocation and export of the capsular polysaccharide, were not significantly activated by VirAG.

Another gene cluster that was up-expressed when *virAG* was overexpressed was *BMAA0747–BMAA0751*. The average level of up-expression for this apparent operon was sevenfold. One of these genes, *bimA* (*BMAA0749*), has been linked to actin tail formation involved in intracellular movement of *B. mallei* in macrophages (Stevens *et al*., 2005a). *BMAA0744–BMAA0727,* 19 genes encoding conserved components of a putative T6S system (see below), were activated an average of ninefold by VirAG (Table 1). The six-gene cluster *BMAA1514–BMAA1518.1* was up-expressed an average of 10-fold when *virAG* was overexpressed (Table 1). *BMAA1517* and *BMAA1518.1* encode an AraC family transcriptional regulator and a two-component response regulator respectively. With the exception of *BMAA1516* which is a remnant of an IS*Bma5* transposon, the remaining genes of this cluster encode unique hypothetical proteins that lack Pfam motifs (Finn *et al*., 2006). About a dozen unlinked genes were also activated at least twofold by VirAG, including *BMA2022*, *BMA2786*, *BMAA1575* and seven genes encoding putative transcriptional regulators: *BMA0308, BMA0785, BMA0812, BMA1872, BMA2049, BMAA0830* and *BMAA1127* (Table 1). The results demonstrated that the VirAG regulon contains ~60 genes, including known, likely, and

Table 1. Expression profiling of *B. mallei* overexpressing *virAG*.

Induction by *virAG*[a]	Gene[b]	Locus tag	Description of gene product[c]
2.2		*BMA0308*	Response regulator
2.8		*BMA0785*	PhoB-like response regulator
2.9		*BMA0812*	GntR family transcriptional regulator
3.3		*BMA1872*	KdpE–like response regulator
6.1		*BMA2022*	Phosphodiesterase in COG0583
4.9		*BMA2049*	LysR-type transcriptional regulator
1.6	*wcbS*	*BMA2288*	Capsular polysaccharide (CPS) biosynthesis protein
1.8	*wcbR*	*BMA2289*	CPS biosynthesis protein
1.9	*wcbQ*	*BMA2290*	CPS biosynthesis protein
1.8	*wcbP*	*BMA2291*	CPS biosynthesis protein
2.5	*wcbO*	*BMA2292*	CPS biosynthesis protein
2.7	*wcbN*	*BMA2293*	CPS biosynthesis protein
2.6	*wcbM*	*BMA2294*	CPS biosynthesis protein
2.7	*gmhA*	*BMA2295*	CPS biosynthesis protein
2.6	*wcbL*	*BMA2296*	CPS biosynthesis protein
2.6	*wcbK*	*BMA2297*	CPS biosynthesis protein
2.5	*wcbJ*	*BMA2298*	CPS biosynthesis protein
2.5	*wcbI*	*BMA2299*	CPS biosynthesis protein
2.5	*wcbH*	*BMA2300*	CPS biosynthesis protein
2.9	*wcbG*	*BMA2301*	CPS biosynthesis protein
2.6	*wcbF*	*BMA2302*	CPS biosynthesis protein
2.6	*gspD*	*BMA2786*	Secretin for Type II secretion
3.5	*folE*	*BMAA0727*	Possible GTP cyclohydrolase I
5.9	*tssN*	*BMAA0728*	Hypothetical protein
4.8	*tssM*	*BMAA0729*	Protein with ubiquitin hydrolase domain (PFAM0443)
2.6	*icmF1*	*BMAA0730*	ImpL/SciS/Aec30/VasK-like protein (COG3523)
4.8	*tssL*	*BMAA0731*	Conserved hypothetical protein (COG3455)
5.0	*tssK*	*BMAA0732*	Aec25/VasE/SciO/ImpJ-like protein (COG3522)
2.1	*tssJ*	*BMAA0733*	Conserved hypothetical protein (COG3521)
8.4	*tssI*	*BMAA0734*	Hypothetical protein
1.6	*tssH*	*BMAA0735*	Hypothetical protein
4.5	*tssF*	*BMAA0736A*	Conserved hypothetical protein (COG1357)
4.5	*tssG*	*BMAA0736B*	Conserved hypothetical protein (COG1357)
11	*vgrG1*	*BMAA0737*	VgrG family protein (COG3501)
8.5	*tssE*	*BMAA0739*	EvpG/ImpH/VasB/Aec22/SciB-like protein (COG3520)
7.6	*tssD*	*BMAA0740*	ImpG/EvpF/VasB/SciC/Aec20-like protein (COG3519)
7.5	*tssC*	*BMAA0741*	Aec19/EvpE-like protein (COG3518)
24	*hcp1*	*BMAA0742*	Hcp/SciM/EvpC-like protein (COG3157)
19.4	*tssB*	*BMAA0743*	EvpB/SciI/ImpC-like protein (COG3517)
31.2	*tssA*	*BMAA0744*	ImpB/EvpA/SciH-like protein (COG3516)
4.6	*virA*	*BMAA0745*	Two-component sensor histidine kinase
25	*virG*	*BMAA0746*	Two-component response regulator
3.8	*bimE*	*BMAA0747*	ImpA-related protein (COG3515)
5.3	*bimD*	*BMAA0748*	Hypothetical protein
2.1	*bimA*	*BMAA0749*	Protein with haemagglutinin domain (PFAM05662)
8.1	*bimC*	*BMAA0750*	Protein with heptosyltransferase domain (PFAM01075)
14.7	*bimB*	*BMAA0751*	Possible N-acetylmuramoyl-L-alanine amidase
3.1		*BMAA0830*	Response regulator
1.8		*BMAA1127*	TctD-like response regulator
3.1		*BMAA1514*	Hypothetical protein disrupted by IS*407*A
4.6		*BMAA1515*	Hypothetical protein
3.6		*BMAA1517*	AraC family transcriptional regulator
23.6		*BMAA1518*	Hypothetical protein
21.5		*BMAA1518.1*	SsrB-like response regulator
2.7		*BMAA1575*	Hypothetical protein

a. RNA from mid-log phase *B. mallei* ATCC 23344 containing pBHR2-*virAG* or pSCRhaB2-*virAG* was labelled, hybridized to whole genome *B. mallei* microarrays, and compared with arrays hybridized with analogous RNA from *B. mallei* ATCC 23344 containing pBHR2 or pSCRhaB2. Only selected relevant data from the ~60 genes significantly induced more than twofold by *virAG* overexpression are shown.

b. Gene names *tssA-N* and *bimB-E* were assigned in this study. Locus tag names were assigned previously (Nierman *et al*., 2004) and were taken from NCBI.

c. Description of the gene product was manually derived from data for each ORF obtained from IMG (http://img.jgi.doe.gov/) and includes homologues of T6S proteins from *E. coli* (Aec), *V. cholerae* (Vas), *Edwardsiella tarda* (Evp), *Salmonella enterica* (Sci) and *Rhizobium leguminosarum* (Imp).

Table 2. Expression profiling of *B. mallei* overexpressing BMAA1517.

Induction by 1517[a]	Gene[b]	Locus tag	Description of gene product[c]
2.2	*folE*	*BMAA0727*	Possible GTP cyclohydrolase I
4.0	*tssN*	*BMAA0728*	Hypothetical protein
2.5	*tssM*	*BMAA0729*	Protein with ubiquitin hydrolase domain (PFAM0443)
1.8	*tssL*	*BMAA0731*	Conserved hypothetical protein (COG3455)
2.0	*tssK*	*BMAA0732*	Aec25/VasE/SciO/ImpJ-like protein (COG3522)
2.1	*tssI*	*BMAA0734*	Hypothetical protein
2.2	*tssF*	*BMAA0736A*	Conserved hypothetical protein (COG1357)
2.1	*tssG*	*BMAA0736B*	Conserved hypothetical protein (COG1357)
4.3	*vgrG1*	*BMAA0737*	VgrG family protein (COG3501)
2.2	*tssE*	*BMAA0739*	EvpG/ImpH/VasB/Aec22/SciB-like protein (COG3520)
2.4	*tssD*	*BMAA0740*	ImpG/EvpF/VasB/SciC/Aec20-like protein (COG3519)
2.3	*tssC*	*BMAA0741*	Aec19/EvpE-like protein (COG3518)
12.3	*hcp1*	*BMAA0742*	Hcp/SciM/EvpC-like protein (COG3157)
8.0	*tssB*	*BMAA0743*	EvpB/SciI/ImpC-like protein (COG3517)
8.9	*tssA*	*BMAA0744*	ImpB/EvpA/SciH-like protein (COG3516)
1.9	*virG*	*BMAA0746*	Two-component response regulator
1.9	*bimE*	*BMAA0747*	ImpA-related protein (COG3515)
2.5	*bimD*	*BMAA0748*	Hypothetical protein
16		*BMAA1517*	AraC family transcriptional regulator
2.7		*BMAA1575*	Hypothetical protein

a. RNA from mid-log phase L-rhamnose induced *B. mallei* ATCC 23344 containing pSCRhaB2-*BMAA1517* was labelled, hybridized to *B. mallei* genome microarrays, and compared with arrays hybridized with analogous RNA from the same strain grown in the absence of L-rhamnose. Analysis of array data was as in Table 1.
b,c. Same as in Table 1.

putative virulence determinants. It also suggests that VirAG is part of a regulatory cascade or network for controlling virulence genes.

BMAA1517 encodes an AraC-type transcriptional regulator whose expression is activated by VirAG. BMAA1517 was previously identified as a potential virulence gene regulator using *in silico* genomic subtraction (M.A. Schell, unpublished) and its full-length gene was inserted into the inducible overexpression vector pSCrhaB2 (Cardona and Valvano, 2005). When whole genome expression profiling of induced cultures of *B. mallei* cells with this plasmid was performed, we found that overexpression of *BMAA1517* by ~16-fold resulted in nearly exclusive up-expression of the genes in the T6S cluster by an average of approximately fourfold (Table 2). These results suggest that transcriptional control of the putative T6S gene cluster is complex and involves *virAG, BMAA1517* and perhaps other *virAG*-controlled regulators (Table 1).

VirAG is not required for capsule production

We hypothesized that reduced expression of some of the VirAG-regulated genes discovered above was responsible for the avirulent phenotype of the *virG* mutant. Because capsular polysaccharide is a major *B. mallei* virulence determinant (DeShazer *et al.*, 2001), we explored the role of *virAG* on capsule production. Immunogold electron microscopy with a monoclonal antibody against the *B. mallei* capsule was performed to detect differences in capsular phenotype caused by inactivation or overexpression of *virAG* (Fig. 1). All three strains: wild type with vector, *virG* mutant and wild-type overexpressing *virAG* reacted with the capsular antibodies and showed a thick and evenly distributed capsule. There was no evidence that overexpression of *virAG* increased capsule production (Fig. 1B), despite the apparent increased expression of some capsule genes (Table 1). Neither a *virG* mutant (Fig. 1C) nor a *virA* mutant (not shown) exhibited evidence of reduced capsule production (Fig. 1C). In addition, there was no noticeable difference in the mucoid phenotype of colonies formed on agar plates by these strains. These results suggest that VirAG is not required for capsule production and that altered expression of the capsule genes is probably not responsible for the reduced-virulence phenotype of the *virG* mutant.

Actin-based intracellular motility requires virAG *and three new linked genes*

The *bimA*-containing gene cluster *BMAA0747–BMAA0751* was strongly activated by *virAG* (Table 1). *BMAA0748–BMAA0751* comprised an apparent operon downstream of *virAG* that is transcribed convergently (Fig. 2). Because of their linkage to *bimA (BMAA0749)*, *BMAA0747*, *BMAA0748*, *BMAA0750* and *BMAA0751* were assigned the gene names *bimE*, *bimD*, *bimC* and *bimB*, respectively (Fig. 2A and Table 1). Mutations were constructed in *bimA*, *bimB*, *bimC* and *bimE* and the corresponding mutants were examined for their ability to form

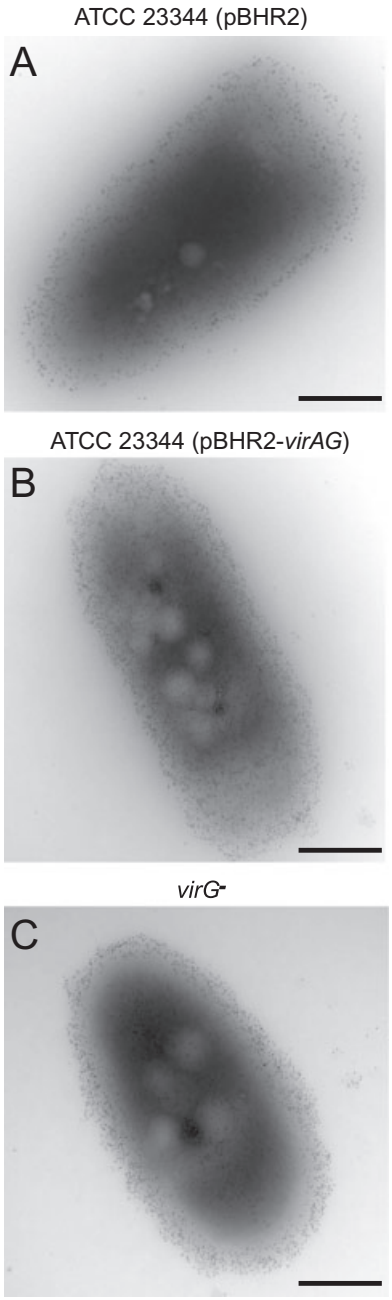

Fig. 1. Immunogold electron microscopy of *B. mallei* strains. Bacteria were reacted with a monoclonal antibody directed against the capsular polysaccharide, washed, and reacted with a goat anti-mouse IgG gold conjugate (5 nm).
A. ATCC 23344 (pBHR2).
B. ATCC 23344 (pBHR2-*virAG*).
C. DDA0746 (*virG*⁻). Bars represent 500 nm.

actin tails in J774.2 murine macrophage-like cells (Fig. 3). Consistent with the report of (Stevens *et al*., 2005a), Fig. 3A illustrates how wild-type *B. mallei* multiplied in the cytosol of the macrophages and spread directly from cell-to-cell by using an actin-based mechanism of motility; Fig. 3B shows how this phenotype required *bimA*. The *bimB*, *bimC*, *bimE* and *virG* mutants also did not form actin tails (Fig. 3C–F) nor did *virA* mutants (data not shown). This demonstrates for the first time that these genes are also required for actin-based motility. These results also suggest that formation of actin tails by *B. mallei* and its regulation is more complex than previously appreciated and that *virAG* is necessary for transcription of *bim* genes inside host cells.

Attempts to construct a mutation in *bimD* were unsuccessful and the role of this gene in actin tail formation is currently unclear. The *bimC* mutant used in this study contains an in-frame deletion and is unlikely to have a polar effect on downstream genes. The *bimB*, *bimA* and *bimE* mutants, on the other hand, harbour EZ::TN<KAN-2> insertions in which the kanamycin resistance gene of the transposon is transcribed in the same orientation as the gene that is disrupted. EZ::TN<KAN-2> does not contain transcriptional terminators and several studies have shown that inactivation by this transposon does not prevent expression of downstream genes (Lin *et al*., 2002; Holm *et al*., 2003; Rhodes *et al*., 2005). Thus, it is unlikely that the EZ::TN<KAN-2> mutations in these strains have a polar effect on the expression of downstream genes.

Actin-based motility is not required for virulence in the hamster model of B. mallei *infection*

Syrian hamsters are a traditional animal model used to study glanders because of their uniform susceptibility to infection, which is characterized by an acute sepsis and the development of exudative granulomas with pronounced necrosis (Miller *et al*., 1948; Ferster and Kurilov, 1982; Fritz *et al*., 1999). The intraperitoneal 50% lethal dose (LD_{50}) is ~10 wild-type cells. In order to determine the relative virulence of the actin tail formation mutants, we infected animals with 10^1–10^4 cells of each strain. All animals infected with wild-type *B. mallei* ATCC 23344 succumbed, usually within 4–5 days, as did animals infected with *B. mallei* SR1 (Ulrich *et al*., 2005), an ATCC 23344 derivative used to construct the *bim* and *virAG* mutants described above (Table 3). In contrast, all animals infected with the *virA* and *virG* mutants survived. When *virAG* was supplied *in trans* and expressed from a constitutive promoter in the *virG*⁻ mutant, there was full complementation, i.e. all animals succumbed (Table 3). This indicated that the *virG* in-frame deletion mutation was not polar and that *virG* was unambiguously required for *B. mallei* virulence in this acute model of infection. Surprisingly, all animals infected with the *bimA*, *bimB*, *bimC* and *bimE* mutants succumbed in a fashion and time frame identical to animals infected with wild-type organisms (Table 3). These results demonstrate that actin-based motility and cell-to-cell spread was not required for

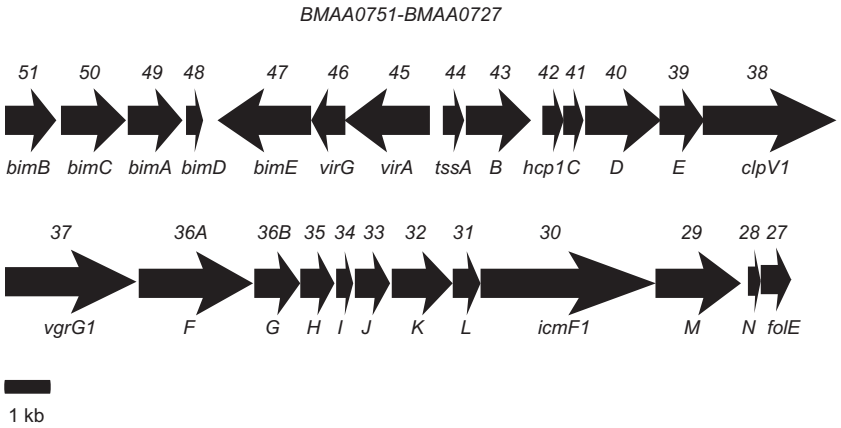

Fig. 2. Genetic map of the *virAG* regulated T6S gene cluster of *B. mallei*. The locus encoding actin-based motility genes (*bim*), *virAG* and T6S genes (*tss*) (chromosome 2 coordinates 771 918–735 641) is shown schematically. The location and direction of transcription of genes are represented by arrows. Locus tags assigned by TIGR are shown above and the gene names assigned in this study and elsewhere (Stevens *et al*., 2005a) are shown below. The genes *BMAA0736A* and *BMAA0736B* were originally annotated as a single gene (*BMAA0736*) harbouring a frameshift mutation (Nierman *et al*., 2004). A scale (in kb) is shown at the bottom.

B. mallei virulence in hamsters. In addition, it is unlikely that lack of actin tail formation is responsible for the reduced virulence phenotype of the *virG* mutant.

Secretion of Hcp1 (BMAA0742) is dramatically enhanced by overexpression of virAG *or BMAA1517*

The *BMAA0744–BMAA0727* gene cluster, whose expression is activated by *virAG* and *BMAA1517*, is located immediately upstream of *virAG* and is transcribed divergently in an apparent operon (Fig. 2). This set of genes has all the hallmarks of a T6SS in that it contains ~15 genes encoding protein families that are associated with components of T6SSs (Das and Chaudhuri, 2003; Schlieker *et al*., 2005; Dudley *et al*., 2006; Mougous *et al*., 2006; Pukatzki *et al*., 2006). Among others these are a ClpV-ATPase/chaperone-like protein (COG0542), an Hcp-like protein (COG3157), an uncharacterized low-

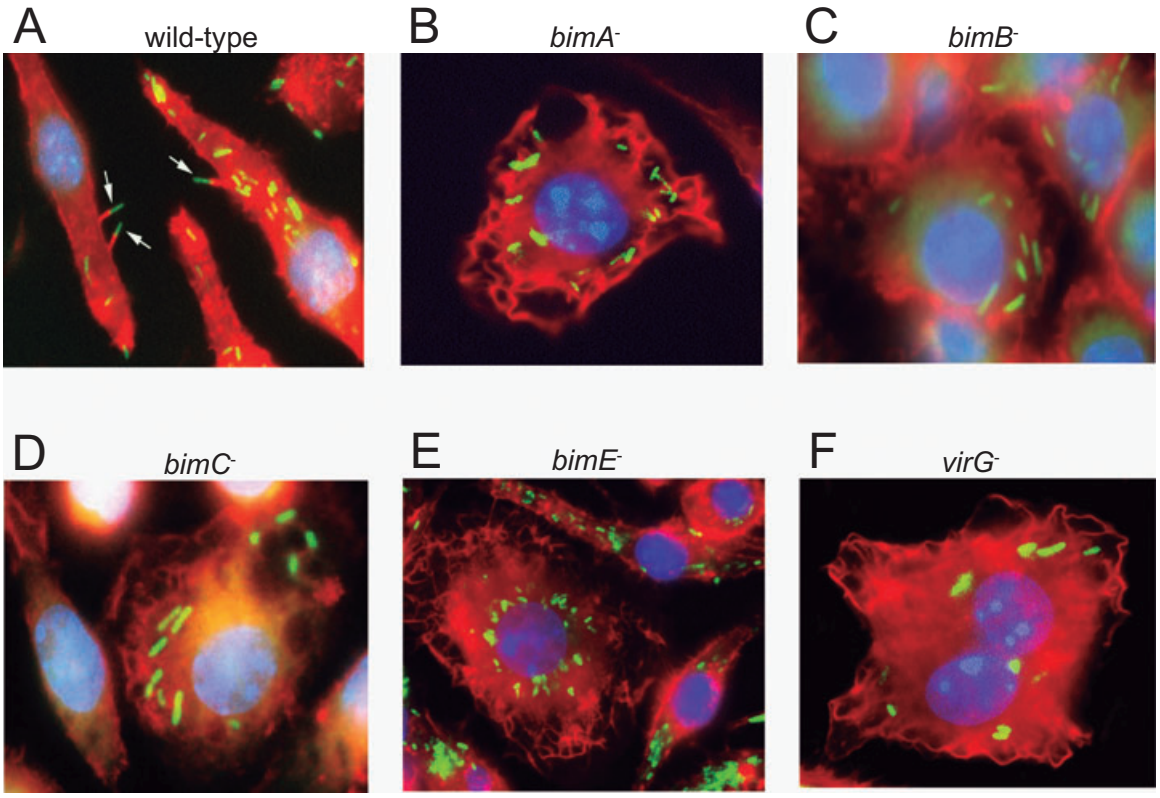

Fig. 3. *Burkholderia mallei* actin-based intracellular motility requires *bimA*, *bimB*, *bimC*, *bimE* and *virG*. Representative micrographs of J774.2 cells infected with (A) SR1 (wild-type), (B) DDA0749 (*bimA*$^{-}$), (C) DDA0751 (*bimB*$^{-}$), (D) DDA0750 (*bimC*$^{-}$), (E) DDA0747 (*bimE*$^{-}$) and (F) DDA0746 (*virG*$^{-}$). *B. mallei* appears green owing to expression of GFP from a modified broad-host-range vector (pBHR4-GFP) and filamentous actin appears red due to staining with Alexa Fluor^{568}-phalloidin. The nucleus was stained blue with 4′,6-diamidino-2-phenylindole (DAPI). The arrows in A show several bacteria with actin tail formation occurring at one pole.

Table 3. Type VI secretion, but not actin-based motility, is required for *B. mallei* virulence in the Syrian hamster model of infection.

	Number of animals that succumbed/total infected[a]			
Strain	10^1 cfu[b]	10^2 cfu	10^3 cfu	10^4 cfu
ATCC 23344	ND	5/5	5/5	5/5
SR1	4/5	5/5	5/5	ND
virA$^-$	ND	0/5	0/5	0/5
virG$^-$	ND	0/5	0/5	0/5
virG$^-$/*virAG*$^+$	5/5	5/5	5/5	ND
bimA$^-$	ND	5/5	5/5	5/5
bimB$^-$	ND	5/5	5/5	5/5
bimC$^-$	ND	5/5	5/5	5/5
bimE$^-$	ND	5/5	5/5	5/5
tssB$^-$	0/5	0/5	0/5	ND
hcp1$^-$	0/5	0/5	0/5	ND
tssD$^-$	0/5	0/5	0/5	ND
tssE$^-$	0/5	0/5	0/5	ND
tssE$^-$/*tssE*$^+$	3/5	5/5	5/5	ND

a. Three groups of five female Syrian hamsters were infected by the intraperitoneal route with a range of 10^1–10^4 cfu for each strain examined. Mortality was recorded daily for 14 days.
b. Colony-forming units.
ND, not determined.

complexity protein (COG1357), an ImpH-like protein (COG3520), and an ImpA-like protein (COG3515) (see Fig. 7 for a complete list). The genes in this cluster were designated *tssA*–*tssN* for type six secretion with the exceptions of *BMAA0742*, *BMAA0738*, *BMAA0737* and *BMAA0730* which were given the names *hcp1, clpV1, vgrG1 and icmF1,* respectively, for consistency with the nomenclature used for other T6SSs. Surprisingly, *B. mallei* and *Burkholderia pseudomallei* have at least three other gene clusters remarkably similar to *tssA*–*tssN* that are likely to be T6SSs (see below).

In order to determine if *virAG* and *BMAA1517* promote protein secretion in *B. mallei* and identify T6SS substrates, we used SDS-PAGE and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to analyse proteins in *B. mallei* culture supernatants, especially looking for Hcp1, the protein encoded in the *B. mallei* T6S gene cluster (Fig. 2) that was similar to Hcp-family proteins that are secreted by T6SSs in *V. cholerae*, *P. aeruginosa*, *Edwardsiella tarda* and enteroaggregative *Escherichia coli* (Dudley *et al*., 2006; Mougous *et al*., 2006; Pukatzki *et al*., 2006; Rao *et al*., 2004). LC-MS/MS analyses showed that Hcp1 was barely detectable in culture supernatants of wild-type *B. mallei*, but when *virAG* was overexpressed, Hcp1 became the major protein present in the culture supernatant. Similar results were obtained when *BMAA1517* was overexpressed. SDS-PAGE analyses of analogous culture supernatants showed that overexpression of *virAG* caused a dramatic increase in the levels of a ~22 kDa polypeptide that was very close to the size predicted for Hcp1 and to a lesser extent polypeptides of ~45 kDa and 15 kDa (Fig. 4). SDS-PAGE and LC-MS/MS analy-

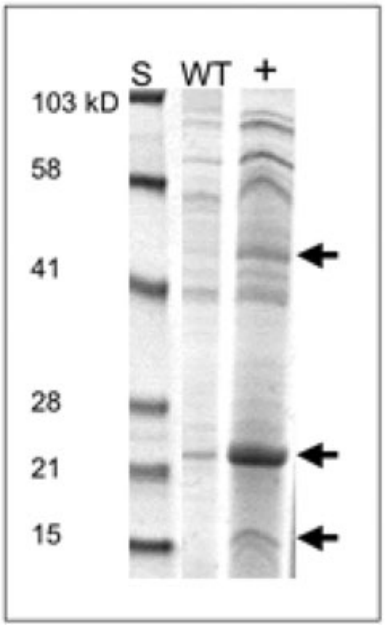

Fig. 4. Comparative SDS-PAGE analysis of culture supernatant from *B. mallei* overexpressing *virAG*. Cultures of wild-type *B. mallei* containing pBHR2 vector (WT) or pBHR2-*virAG* (+) were grown for 36 h in 3% glycerol containing 3% dialysed casamino acids and 3% dialysed yeast extract. Cells were removed by centrifugation and the filter sterilized culture supernatant concentrated ~200-fold by ultrafiltration (10 kDa cut-off). An equivalent of 5 ml of original culture was loaded on a 4–20% gradient SDS-PAGE gel (Pierce 25204). After 2 h, gel was stained with colloidal Coomassie blue. Arrows indicate proteins that were overproduced in ATCC 23344 (pBHR2-*virAG*) relative to ATCC 23344 (pBHR2). Migration of molecular weight standards (S), in kDa, are indicated on the left.

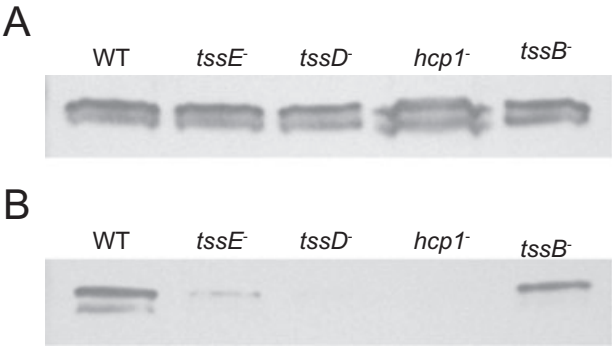

Fig. 5. Immunoblot analysis of V5-tagged Hcp1 in *B. mallei* wild-type and T6S mutants. Cell pellet proteins (A) and supernatant proteins (B) were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with an anti-V5-HRP antibody. WT, SR1 (pBHR2-*hcpV5*/*virAG*); *tssE*⁻, DDA0739 (pBHR2-*hcpV5*/*virAG*); *tssD*⁻, DDA0740 (pBHR2-*hcpV5*/*virAG*); *hcp1*⁻, DDA0742 (pBHR2-*hcpV5*/*virAG*); and *tssB*⁻, DDA0743 (pBHR2-*hcpV5*/*virAG*).

ses showed no evidence of leakage of cytoplasmic or periplasmic proteins and thus these results strongly suggest that Hcp1 is secreted *in vitro* by *B. mallei.* The lack of a signal sequence on Hcp1 and correlation between the transcriptional activation of the T6S gene cluster and levels of Hcp1 in the culture supernatant further suggest that Hcp1 is a secreted substrate of this T6S.

tssE, tssD *and* hcp1 *are required for secretion of V5-tagged Hcp*

We constructed strains harbouring in-frame deletions in *tssB*, *hcp1*, *tssD* and *tssE*, four genes encoding proteins that are highly conserved in T6SSs and analysed them for their ability to secrete a Hcp1 derivative harbouring a C-terminal V5 epitope tag (HcpV5). The *hcpV5* gene and *virAG* were coexpressed from a constitutive promoter on pBHR2, cell pellets and supernatants were collected, and immunoblots performed with an anti-V5-HRP monoclonal antibody. Figure 5A shows that HcpV5 was present in the cell pellets of wild-type and all T6S mutants, demonstrating that the V5-tagged Hcp1 protein was similarly produced by all strains. The HcpV5 protein migrated as a doublet on SDS-PAGE (Fig. 5) possibly due to a post-translational modification of some of the HcpV5 protein. While the wild-type strain was able to secrete HcpV5 into the supernatant, the *tssE*, *tssD* and *hcp1* mutants secreted little or no HcpV5 into the supernatant (Fig. 5B). In contrast, the *tssB* mutant secreted HcpV5 almost as well as wild-type organisms (Fig. 5B). These results clearly demonstrate that this *B. mallei* T6SS is functional and that *tssE*, *tssD* and *hcp1* are probably required for secretion of Hcp1. In contrast *tssB* was apparently not required for the secretion of Hcp1 and thus may not be a secreton component of this T6SS.

T6S is required for virulence in hamsters

The *tssB*, *hcp1*, *tssD* and *tssE* mutants were examined for their relative virulence in the hamster model of infection (Table 3). The LD_{50} of these mutants was $> 10^3$ colony-forming units (cfu) and no hamsters infected with any of the T6S mutants succumbed. In addition, the *tssE*⁻ strain was complemented when *tssE* was supplied *in trans* on a broad-host-range plasmid with a constitutive promoter (Table 3), demonstrating that the Δ(*tssE*) mutation was not polar and that *tssE* was critical for virulence likely because it was also required for secretion of Hcp1. Interestingly, the *tssB*⁻ mutant was avirulent even though it was not required for T6S-mediated secretion of HcpV5 (Fig. 5B). We speculate that TssB may be another substrate of this T6S. Taken together, the results demonstrate that T6S is a major virulence determinant in *B. mallei* and that the T6S gene cluster is probably responsible for the avirulent phenotype of the *virG* mutant.

Hcp is produced during B. mallei *infection in several hosts*

His-tagged HcpV5 protein was purified and used in immunoblot experiments probed with glanders sera from mice, a horse (Scholz *et al.*, 2006) and a human (Srinivasan *et al.*, 2001). The SDS-PAGE and immunoblot images shown in Fig. 6A and B demonstrate that the purified HcpV5 protein was homogeneous, had the correct predicted size, and reacted with the anti-V5-HRP antibody. To determine if Hcp1 was produced *in vivo*, sera were collected from a group of five mice chronically infected with *B. mallei* and a group of control mice. The pooled sera from the control mice did not react with HcpV5, but the pooled sera from the chronically infected mice did react with HcpV5 (Fig. 6C and D). Similarly, serum from a naturally infected horse with glanders reacted with HcpV5, whereas serum from an uninfected horse did not (Fig. 6E and F). Serum obtained from a human with laboratory-acquired glanders also recognized the HcpV5 protein (Fig. 6H), but pre-infection serum from the same patient did not (Fig. 6G). These results clearly demonstrate that Hcp1 is expressed during infection in multiple hosts.

Six distinct T6S gene clusters are variably present in B. pseudomallei, B. mallei *and* Burkholderia thailandensis

Using the integrated microbial genomes site (IMG; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi), we searched the genome of *B. mallei* (and the closely related bacteria *B. pseudomallei* and *B. thailandensis*) for open reading frames (ORFs) assigned to COG protein families whose

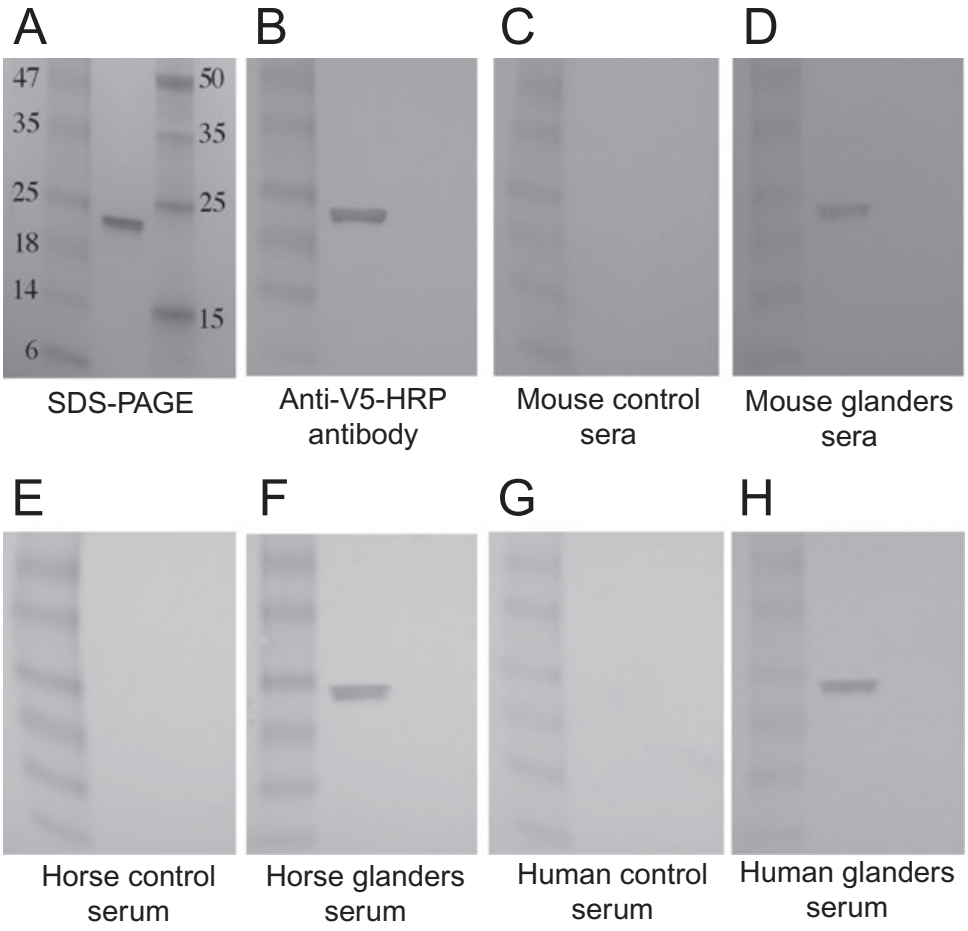

Fig. 6. Immunoblot analysis of recombinant HcpV5 protein using glanders antisera. Proteins were separated by SDS-PAGE (A) and immunoblots were reacted with an anti-V5-HRP antibody (B), pooled sera from control mice (C), pooled sera from chronically infected mice (D), control horse serum (E), horse glanders serum (F), human control serum (G) and human glanders serum (H). Lanes 1, BenchMark Pre-stained Protein Ladder (Invitrogen); lanes 2, recombinant HcpV5; lanes 3, Perfect Protein Markers, 15–150 kDa (Novagen). The apparent molecular masses, in kDa, of the protein markers are shown in Fig. 6A.

members are conserved components of T6SSs (such as COG3517, COG3519 and COG3522). We found three additional putative T6S gene clusters in *B. mallei*, each containing ~15 T6SS-conserved genes from 14 different protein families and approximately three hypothetical proteins (Table 4 and Fig. 7). In *B. pseudomallei*, the presumed parent of the *B. mallei* clone, and in *B. thailandensis*, a close relative of *B. pseudomallei*, we found six and five complete T6S gene clusters, respectively (Table 4). The relative gene order in each T6S cluster is similar and genes in the following COG families form 'three-gene modules' at distinct locations in each cluster: COG3516/COG3517/COG3157, COG3518/COG3519/COG3520 and COG3521/COG3522/COG3455 (see boxes in Fig. 7). The co-occurrence of genes on genomes has been shown to indicate a func-

Table 4. Type VI secretion gene clusters present in *B. mallei*, *B. pseudomallei* and *B. thailandensis*.

Cluster	*B. mallei*	*B. pseudomallei*	*B. thailandensis*
1	BMAA0744–0729	BPSS1496-1512	BTH_II0870-0854
2	BMAA0438–0455	BPSS0515-0533	BTH_II1902-1883
3	BMAA0396–0412	BPSS2107-2091	BTH_II0251-0267
4	BMAA1897–1915	BPSS0166-0185	Absent
5	Absent	BPSS0095-0116	BTH_II0119-0140
6	BMA2826–2833[a]	BPSL3097-3111	BTH_I2954-2968

a. The last seven genes, encoding COG3157-COG3455, are missing in *B. mallei* ATCC 23344.

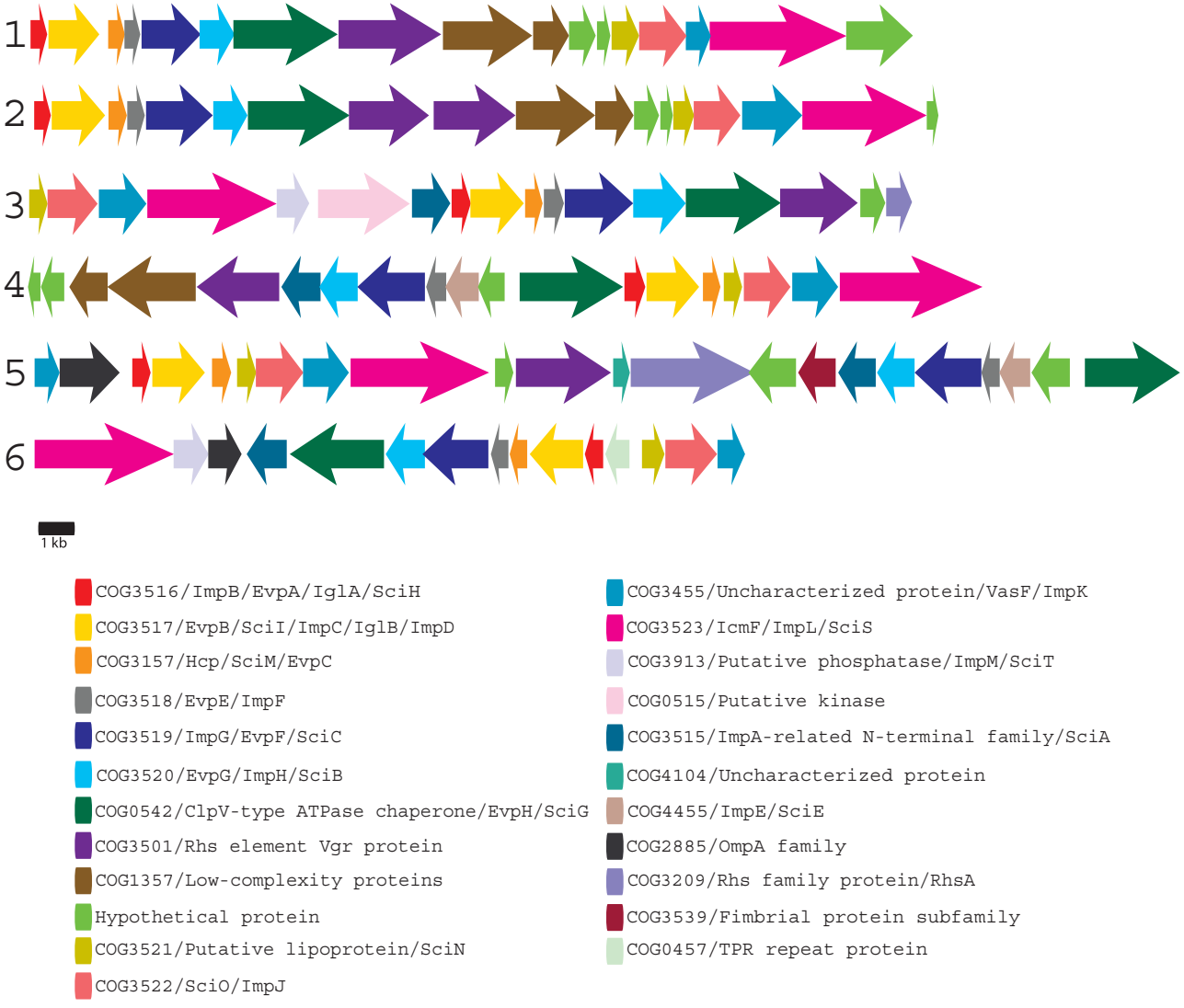

Fig. 7. Six distinct T6S gene clusters are variably present in *B. pseudomallei, B. mallei and B. thailandensis.* The numbers at the left of each genetic locus represent the T6S cluster numbers described in the text and in Table 4. The location and direction of transcription of T6S genes are represented by arrows and are colour-coded based on the COG classification of the proteins they encode (Tatusov *et al.*, 2003). A brief description of the protein products, or a listing of homologous T6S proteins from *V. cholerae* (Vas), *Edwardsiella tarda* (Evp), *Salmonella enterica* (Sci), *Francisella tularensis* (Igl) and *Rhizobium leguminosarum* (Imp), are also shown. Proteins that do not have a COG classification are shown as hypothetical proteins. A scale (in kb) is shown at the bottom.

tional association between the proteins they encode (Snel *et al.*, 2000), suggesting that the proteins encoded by each of these three-gene modules may physically interact to form substructures of the T6S apparatus.

While clusters 1–3 appear to comprise a single transcriptional unit, clusters 4–6 are divided into two to three transcriptional units (Fig. 7). Clusters 1–3 are present in all three species and in general show very high sequence conservation (> 80%), while cluster 4 is unique to pathogenic *B. mallei* and *B. pseudomallei* (Table 4). Cluster 5 is present in both *B. pseudomallei* and *B. thailandensis,* but missing from *B. mallei*. Cluster 5 differs from the other clusters in that it harbours a gene that encodes a protein involved in fimbrial biogenesis (COG3539; Fig. 7). Cluster 6, the only one on chromosome 1, appears to be widely conserved among many soil-dwelling members of the *Burkholderia* and *Ralstonia*. *B. mallei*, an obligate mammalian pathogen, contains a deletion that spans the last seven genes of this cluster (Table 4 and Fig. 7).

In terms of G + C content, G + C content at third position in codons, and codon usage, *B. mallei* clusters 1–4 are not significantly distinct from the rest of chromosome 2 and thus do not appear to be recently or horizontally

transferred (Fig. S1). *B. mallei* SR1 contains a deletion mutation that spans the cluster 2 T6S genes (Nierman *et al*., 2004; Ulrich *et al*., 2005), but this strain is still highly virulent in hamsters (Table 2). This clearly demonstrates that in contrast to the cluster 1 T6S genes described here, the cluster 2 T6S genes are not required for *B. mallei* virulence. The role of T6S clusters 3 and 4 in *B. mallei* virulence is currently unknown as is the role of all the T6S clusters in *B. pseudomallei* and *B. thailandensis.*

Discussion

Here we demonstrated that the *B. mallei* VirAG two-component regulatory system activates transcription of a regulon consisting of ~60 genes (Table 1). About 15 of these are contiguous genes that are part of the 24-gene capsular polysaccharide gene cluster. Despite this activation, excess capsule was not detected (Fig. 1). More importantly *virG* mutants appeared to have wild-type capsule production. The VirAG-regulated capsule genes are predicted to encode for production of 6-deoxy-β-D-*manno*-heptopyranose, the only sugar in the capsular polysaccharide of *B. mallei* (DeShazer *et al*., 2001). *bimC,* encoding a putative heptosyltransferase, was also activated by *virAG* and is required for actin-based intracellular motility of *B. mallei. bimC* is adjacent to *bimA,* a gene encoding an autotransported protein involved in forming the actin tails needed for actin-based motility (Stevens *et al*., 2005a). In *E. coli*, the autotransported adhesins TibA and AIDA-I are glycosylated by the adjacently encoded heptosyltransferases TibC and AAH (Lindenthal and Elsinghorst, 1999; Benz and Schmidt, 2001). TibA and AIDA-I are not functional unless they are glycosylated with heptosyl residues that are diverted from the *E. coli* lipopolysaccharide biosynthesis pathway. We demonstrated here that a *B. mallei bimC* mutant does not polymerize actin and hypothesized that BimA maybe glycosylated by BimC to be active. We further hypothesized that activation of part of the capsule gene cluster by VirAG serves to increase the pool of 6-deoxy-β-D-*manno*-heptopyranosyl residues available for glycosylation of BimA. Consistent with this proposal, a capsule-like substance on the surface of *B. mallei* and *B. pseudomallei* (called antigen 8) was isolated and reported to be a glycoprotein with a carbohydrate moiety identical in structure to the capsular polysaccharide of *B. mallei* (Piven *et al*., 1996; Khrapova *et al*., 1998; Samygin *et al*., 2001). Further studies are necessary to determine if BimA is glycosylated and is related to antigen 8.

Actin-based motility is used by intracellular bacteria to spread from cell-to-cell without triggering the humoral immune response (Carlsson and Brown, 2006). The ability to polymerize actin is an important virulence factor for both *Listeria monocytogenes* and *Shigella sonnei* (Brundage *et al*., 1993; Orr *et al*., 2005). Recently, it was demonstrated that the *B. mallei* BimA protein could induce actin polymerization within macrophages, and could restore the actin-based motility defect of a *B. pseudomallei bimA* mutant (Stevens *et al*., 2005a). In our study, we constructed a *B. mallei bimA* mutant and found that it could not polymerize actin (Fig. 3B). We also identified five additional genes, *bimB*, *bimC*, *bimE*, *virA* and *virG,* required for actin tail formation and found that transcription of *bimA-E* was strongly activated by *virAG,* thereby explaining the actin-tail defective phenotype caused by mutations in *virAG*. The biochemical role of other Bim proteins in actin polymerization is unknown, except for BimB, a putative N-acetylmuramoyl-L-alanine amidase, which possibly could function in localized cell wall breakdown needed for BimA export.

Before this study, actin tail formation had been implicated as a *B. mallei* virulence determinant, but never confirmed in animals. Surprisingly, we found that *bimA*, *bimB*, *bimC* and *bimE* mutants were still virulent in hamsters, suggesting that actin-based motility is not a critical virulence determinant in this glanders model. *B. mallei* is a host-adapted pathogen which evolved from a *B. pseudomallei*-like ancestor by massive IS-element facilitated gene loss and appears to be continuing in its evolution/adaptation using mutation to eliminate or inactivate genes that are not essential for a successful host–parasite interaction (Nierman *et al*., 2004; Kim *et al*., 2005). It is puzzling that the actin polymerization phenotype has been maintained by this pathogen if it is not required for *in vivo* survival. It is possible that actin-mediated intercellular motility enhances virulence only to a limited extent in the acute hamster model of infection. Evasion of the immune system using actin-based motility may be more critical in 'naturally infected' hosts and experiments using the mouse model of chronic infection may provide insight into this anomaly.

The overexpression of *virAG* strongly activated expression of the T6S gene cluster located immediately upstream of *virAG* (Table 1 and Fig. 2). Almost every gene in this T6S pathway was up-expressed at least threefold, and most were activated more than fivefold. Overexpression of *BMAA1517*, encoding an AraC-type activator, also increased transcription of these T6S genes (Table 2). The hierarchy and relationship of these regulators to each other in T6S regulation was unclear. Therefore, we placed the pBHR2-*virAG* overexpression plasmid into a *BMAA1517* deletion mutant and compared expression profiles with its plasmidless parent. Strong up-expression of *BMAA0729–BMAA0752,* averaging approximately eightfold, was observed (data not shown). In fact, the expression profile of the *BMAA1517* mutant with pBHR2-*virAG* was largely identical to that of wild-type *B. mallei* overexpressing *virAG*, including strong up-expression of

bimBCDE. These data suggest that *virAG* and *BMAA1517* independently control transcription of this T6S cluster, which is poorly expressed *in vitro*. Presumably, VirAG and BMAA1517 sense specific conditions in the host environment, and in response, activate the cluster 1 T6S genes (Fig. 7). Although Tuanyok *et al*. (2005) recently showed that the T6S genes *hcp1* and *tssC* were highly induced by low iron, all other T6S genes in cluster 1 were unaffected, thus ruling out iron as a signal for VirAG or BMAA1517. The stringent and complex transcriptional regulation of the *B. mallei* T6S gene cluster is similar to that observed for the T6S gene clusters in other pathogens (Bladergroen *et al*., 2003; Zheng *et al*., 2005; Dudley *et al*., 2006; Mougous *et al*., 2006; Pukatzki *et al*., 2006). Thus, it is likely that optimal timing of T6S expression is of the utmost importance during the host–pathogen interaction and that premature or inappropriate expression of this secretion system may negatively impact *in vivo* survival.

The T6S encoded in cluster 1 is an essential virulence determinant for *B. mallei* in hamsters and likely many other hosts because mutants that are avirulent in hamsters are also avirulent in other animal models, including the horse (DeShazer *et al*., 2001; Lopez *et al*., 2003; Ulrich and DeShazer, 2004; Ulrich *et al*., 2004). To our knowledge, this is the first time that a T6S mutant of a mammalian pathogen has been shown to be deficient in both secretion and virulence in a mammalian model of infection. We demonstrated that Hcp1 was secreted by the T6S system and that *tssD*, *tssE* and *hcp1* were required for secretion of an epitope-tagged version of Hcp1 (i.e. HcpV5). While Hcp1 was necessary for HcpV5 secretion, HcpV5 was unable to promote its own secretion in an *hcp1* mutant (Fig. 5B). Because Hcps are hypothesized to be involved in protein–protein interactions required for assembly and function of the T6S apparatus (Mougous *et al*., 2006; Pukatzki *et al*., 2006), it may be that the C-terminal V5 tag interferes with its ability to interact with other proteins in the T6S apparatus and allow its own secretion. The exact function of Hcp1 during infection is currently unknown, but it was absolutely required for virulence in hamsters (Table 3). Moreover, Hcp1 is secreted *in vivo* and is immunogenic in mice, horses and humans (Fig. 6). Thus, Hcp1 may be useful as a vaccine candidate and/or as a diagnostic reagent for both human and animal glanders infections and studies are in progress to address these issues.

There are multiple Hcps in *B. mallei*, *B. pseudomallei* and *B. thailandensis*, one in each of the T6SS gene clusters (Fig. 7). While the amino acid sequences of most T6S components are highly conserved (> 85%) within each species, the sequences of the Hcp-family proteins show much more diversity implying differences in specificity or function. Amino acid sequence variability among various Hcps may also result from immune-response-driven sequence variation occurring in the non-conserved regions identified by Mougous *et al*., 2006). However, alignment of BMAA0729 with Hcps from the *Burkholderia-Ralstonia* clade shows it has < 30% amino acid identity with any other member and also has two unique 5-residue insertions. Phylogenetic trees constructed from alignments show BMAA0729 does not cluster with any other Hcp. Finally, wide sequence variations in Hcps are still observed when comparing Hcp sequences of non-pathogenic *Burkholderia-Ralstonia*. The observation that genomes of *Photorhabdus luminescens* and *Yersinia pestis* CO92 have nine different Hcp-like proteins for only five T6S clusters further supports this suggestion.

In addition to Hcp-1 and Hcp-2, *V. cholerae* also secretes VgrG-1, VgrG-2 and VgrG-3 via the T6S pathway (Pukatzki *et al*., 2006). Although we unambiguously identified one protein, Hcp1, in culture supernatants of *B. mallei* overexpressing *virAG*, it is highly probable that other proteins transit the T6S apparatus and our SDS-PAGE analysis (Fig. 4) shows hints of these proteins. More sensitive mass spectrometry methods are currently being employed to detect these putative effector proteins and recently identified VgrG1 (BMAA0737), a VgrG-like protein, in culture supernatants of *B. mallei* overexpressing *virAG*.

Interestingly, *tssB* was not required for HcpV5 secretion even though it was necessary for hamster virulence (Table 3 and Fig. 5). One possible explanation for this phenotype is that TssB is itself a T6S-secreted effector-like protein that is required for hamster pathogenesis. Another potential secreted effector encoded by this T6S cluster is TssM, a protein containing a ubiquitin-specific proteinase domain (Peptidase_C19E subfamily). Because ubiquitin-tagging of proteins does not occur in prokaryotes, it is possible that TssM is secreted into host cells where it could interfere with the ubiquitin-proteasome degradation system or with non-proteosomal ubiquitin functions like trafficking, endocytosis, gene expression, gene silencing and kinase activation. This possibility is very intriguing, as ubiquitin-proteasome degradation system is involved in signalling that affects inflammation and other immune responses in mammals (Wang and Maldonado, 2006).

Searches of the ~600 microbial genomes (~450 species) in IMG for all proteins assigned to T6S-associated COG families showed T6SSs are restricted to a minority (85) of proteobacterial species with most harbouring one or two T6Ss. Only 12 species from four genera have three or more T6S clusters with many, but not all, being pathogens. *B. mallei, B. pseudomallei, B. thailandensis, Y. pestis and P. luminescens* are outstanding in that they have between four and six T6S clusters. Interestingly, cluster *BMAA0438–0455* of *B. mallei* is very similar in sequence and organization to

one of the T6S clusters of *Y. pestis* CO92 (*YPO0499–0515*) with some components including the Hcp-homologue and TssB-homologue showing > 65% amino acid identity. None of the other 450 species in the IMG (with the exception of *Yersinia pseudotuberculosis*) has a T6S cluster orthologous to *BMAA0438–0455,* suggesting this cluster is unique to *B. mallei*, *B. pseudomallei*, *B. thailandensis*, *Y. pestis* and *Y. pseudotuberculosis*.

Analysis of the G + C content, G + C content at codon site 3, and codon usage of the four *B. mallei* T6S gene clusters suggest that they were not recently acquired by lateral transfer (Fig. S1). It appears that the multiple T6S gene clusters arose from 'ancient' duplication events and that the genes subsequently diverged to be functionally distinct from each other. All *B. mallei* T6S gene clusters are on chromosome 2, which has been described as a lifestyle-determining replicon important for niche adaptation (Nierman *et al*., 2004; Chain *et al*., 2006), implying that they all may play a role in the interaction of this highly adapted pathogen with its hosts. We showed here that cluster 1 is essential for virulence in *B. mallei*, but the role of the other clusters is unclear. The phenotype of the T6S cluster 1 mutants suggest components of these other T6S clusters cannot cross complement, although this could be due to insufficient expression. The role of these clusters and others comprising the 6 T6SS of *B. pseudomallei* and those in *B. thailandensis* and other bacteria with multiple T6SSs provides much work for the future.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 5. *E. coli* was grown at 37°C on Luria–Bertani (LB) agar (Lennox L agar) or in LB broth (Lennox L broth). *B. mallei* strains were grown at 37°C on LB agar or in LB broth containing 4% glycerol (LBG). When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin (Ap), 50 μg of carbenicillin (Cb), 15 μg of gentamicin (Gm), 25 μg of streptomycin (Sm), 12.5 μg of tetracycline (Tc), 25 μg of kanamycin (Km) and 50 μg of trimethoprim (Tp) per ml for *E. coli* and 15 μg of polymyxin B (Pm), 5 μg Gm, 5 μg of Km and 75 μg of Tp per ml for *B. mallei*. For induction studies, L-rhamnose was added to a final concentration of 0.5% and isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Gene replacement experiments with *B. mallei* SR1 were performed using the *sacB*-based vector pGRV2, as previously described (Ulrich *et al*., 2005). All manipulations with *B. mallei* were carried out in class II microbiological safety cabinets located in designated biosafety level 3 (BSL-3) laboratories.

DNA manipulation and plasmid conjugations

Restriction enzymes, shrimp alkaline phosphatase, and T4 DNA ligase were purchased from Roche Molecular Biochemicals and were used according to the manufacturer's instructions. When necessary, the End-It DNA End-Repair Kit (Epicentre) was used to convert 5′- or 3′-protruding ends to blunt-ended DNA. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III kit (Q·BIOgene). Bacterial genomic DNA was prepared by a previously described protocol (Wilson, 1987). Plasmids were purified from overnight cultures by using Wizard Plus SV Minipreps (Promega). *In vitro* mutagenesis of recombinant plasmids with EZ::TN<KAN-2> was performed according to the instructions provided by the manufacturer (Epicentre). The locations of EZ::TN<KAN-2> insertion sites were determined by sequencing the DNA flanking the insertions sites with provided primers. Recombinant derivatives of pGRV2, pBHR2 and pSCrhaB2 (Table 5) were electroporated into *E. coli* S17-1 (12.25 kV cm^{-1}) and conjugated with *B. mallei* for 8 h, as described elsewhere (DeShazer *et al*., 1997; Ulrich *et al*., 2005). Pm was used to counter-select *E. coli* S17-1.

Polymerase chain reaction (PCR) amplifications

Deoxyribonucleotide primers used in PCR reactions are shown in Table 6. PCR products were sized and isolated using agarose gel electrophoresis, cloned using the pCR2.1-TOPO TA Cloning Kit (Invitrogen), and transformed into chemically competent *E. coli* TOP10. PCR amplifications were performed in a final reaction volume of 100 μl containing 1X *Taq* PCR Master Mix (Qiagen), 1 μM oligodeoxyribonucleotide primers and approximately 200 ng of genomic DNA. PCR cycling was performed using a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research) and heated to 97°C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s and 72°C for 1 min) and one cycle at 72°C for 10 min. For PCR products greater than 1 kb, an additional 1 min per kb was added to the extension time.

Expression profiling experiments

A whole genome PCR amplicon DNA microarray for *B. mallei* ATCC 23344 was fabricated and used as previously described (Nierman *et al*., 2004). Total RNA was isolated from ATCC 23344 (pBHR2-*virAG*) and ATCC 23344 (pBHR2) at the mid-log phase of growth. The OD_{600} of the samples at harvest were ~0.6. Briefly, strains were grown for 8 h in 125 ml disposable Erlenmeyer flasks containing 25 ml of LBG with Km. Five millilitres of culture were processed per 1 ml of TRIzol Reagent (Invitrogen) or RNA protect/RNeasy (Qiagen) following the manufacturer's protocol. The RNAs were labelled and hybridized to the microarray using ATCC 23344 (pBHR2) RNA as the reference. Raw microarray data were generated with TIGR Spotfinder, and then normalized with Lowess, flip-dye analysis and triplicate spot intensity data in MIDAS (Saeed *et al*., 2003). Log2 conversion was performed using TMeV (Saeed *et al*., 2003). Data from four different experiments were analysed for significance using SAM (Tusher *et al*., 2001), averaged, and converted to fold induction ratios using Excel. All values are from a dataset with a >95% confidence level and a false discovery rate of < 0.5%. Similar experiments were also conducted using ATCC 23344 (pSCRhaB2-*virAG*) and ATCC

Table 5. Plasmids and strains used in this study.[a]

Strain or plasmid	Relevant characteristics[b]	Reference
E. coli		
TOP10	General cloning and blue/white screening	Invitrogen
Origami(DE3)	D3 lysogen, Km^R, Sm^R, Tc^R	Novagen
S17-1	Mobilizing strain, transfer genes of RP4 integrated on chromosome, Sm^R, Tp^R, Pm^S	Simon *et al.* (1983)
B. mallei		
ATCC 23344	Type strain, isolated in 1944 from a human case of glanders, Pm^R, Gm^S, Km^S, Tp^S	Nierman *et al.* (2004)
SR1	ATCC 23344 sucrose-resistant derivative, Δ(*BMAA0437–BMAA0497*)	Ulrich *et al.* (2005)
DDA0739	SR1 derivative, Δ(*tssE*)	This study
DDA0740	SR1 derivative, Δ(*tssD*)	This study
DDA0742	SR1 derivative, Δ(*hcp1*)	This study
DDA0743	SR1 derivative, Δ(*tssB*)	This study
DDA0745	SR1 derivative, *virA*::EZ::TN<KAN-2>, Km^R	This study
DDA0746	SR1 derivative, Δ(*virG*)	This study
DDA0747	SR1 derivative, *bimE*::EZ::TN<KAN-2>, Km^R	This study
DDA0749	SR1 derivative, *bimA*::EZ::TN<KAN-2>, Km^R	This study
DDA0750	SR1 derivative, Δ(*bimC*)	This study
DDA0751	SR1 derivative, *bimB*::EZ::TN<KAN-2>, Km^R	This study
Plasmids		
pCR2.1-TOPO	3.9 kb TA vector, pMB1 *oriR*, Km^R, Ap^R	Invitrogen
pCRT7/CT-TOPO	2.7 kb TA vector, allows C-term fusions to V5 eptitope and 6xHis tag under control of a T7 promoter, pMB1 *oriR*, Ap^R	Invitrogen
pUCP30T	Broad-host-range cloning vector, pRO1600 *oriR*, pMB1 *oriR*, *lacZα*, *oriT*, Gm^R	Schweizer *et al.* (1996)
pGRV2	*sacB*-based gene replacement vector, Gm^R	Ulrich *et al.* (2005)
pCR2.1-*BMAA1517*	pCR2.1-TOPO containing S1520F/S1520R PCR product	This study
pEZZ 18	Protein A gene fusion vector, *spa-lacZ'*, Ap^R	Amersham Biosciences
pTn*Mod*-OGm′	Minitransposon vector, pMB1 *oriR*, *oriT*, Tn*5 tnp*, Gm^R	Dennis and Zylstra (1998)
pQBI T7-GFP	GFP expression vector, $lacI^q$, Ap^R	Quantum Biotech.
pCR2.1-GFP	pCR2.1-TOPO containing GFP amplified from pQBI T7-GFP with GFP-up/GFP-dn	This study
pCR2.1-*virAG*	pCR2.1-TOPO containing AT14/AT15 PCR product	This study
pCRT7/CT-A0742	pCRT7/CT-TOPO containing BMAA0742-up/BMAA0742-dn PCR product	This study
pBHR1	Broad-host-range cloning vector, pBBR1 *oriR*, *oriT*, Cm^R, Km^R	MoBiTec
pBHR2	pBHR1 derivative containing the NheI-ClaI insert from pEZZ 18 cloned into the DraI-digested Cm^R gene, *spa-lacZ'*, Cm^S, Km^R	This study
pBHR3	pBHR2 derivative with SacI Gm^R gene from pTn*Mod*-OGm′ inserted into SwaI site, Gm^R, Km^R	This study
pBHR4	pBHR3 derivative lacking the 1240 bp PstI fragment containing Km^R, Gm^R, Km^S	This study
pBHR2-*virAG*	pBHR2 containing SpeI–XbaI insert from pCR2.1-*virAG* cloned into XbaI site	This study
pBHR2-*hcp*/*virAG*	pBHR2-*virAG* containing XbaI–DraI insert from pCRT7/CT-A0742 cloned into EcoRV site	This study
pBHR4-GFP	pBHR4 containing EcoRI insert from pCR2.1-GFP	This study
pSCrhaB2	Broad-host-range expression vector containing rhamnose-inducible promoter, *oriR*, *oriT*, Tp^R	Cardona and Valvano (2005)
pSCrhaB2-*BMAA1517*	pSCrhaB2 containing XbaI–HindIII insert from pCR2.1-*BMAA1517*	This study
pSCRhaB2-*virAG*	pSCRhaB2 containing virAG_3 and virAG_5 PCR product	This study
pCR2.1-A0739	pCR2.1-TOPO containing 39-up/39-dn PCR product	This study
pCR2.1-A0740	pCR2.1-TOPO containing 40-up/40-dn2 PCR product	This study
pCR2.1-42	pCR2.1-TOPO containing 42-up/42-dn PCR product	This study
pCR2.1-A0743	pCR2.1-TOPO containing IGR1/43-dn PCR product	This study
pCR2.1-ΔA0739	pCR2.1-A0739 containing a deletion of the 135 bp SalI fragment internal to *tssE*	This study
pCR2.1-ΔA0740	pCR2.1-A0740 containing a deletion of the 852 bp Eco47III fragment internal to *tssD*	This study
pCR2.1-ΔA0743	pCR2.1-A0743 containing a deletion of the 159 bp AatII fragment internal to *tssB*	This study
pUCP30T-42	pUCP30T containing EcoRI insert from pCR2.1–42	This study
pUCP30T-Δ42	pUCP30T-42 containing a deletion of the 156 bp NarI–SfuI fragment internal to hcp1	This study
pCRT7/CT-AT1112	pCRT7/CT-TOPO containing AT11/AT12 PCR product	This study
pCRT7/CT-AT1112ΔE	pCRT7/CT-AT1112 derivative containing a deletion of the 210 bp EcoRI fragment internal to virG	This study
pCR2.1-AT12	pCR2.1-TOPO containing AT1/AT2 PCR product	This study
pDDA0751-3	pCR2.1-AT12 containing EZ::TN<KAN-2> transposon within *bimB*	This study
pCRT7/CT-AT34	pCRT7/CT-TOPO containing AT3/AT4 PCR product	This study
pCRT7/CT-AT34ΔE	pCRT7/CT-AT34 derivative containing a deletion of the 540 bp EcoRI fragment internal to *bimC*	This study
pCR2.1-AT56	pCR2.1-TOPO containing AT5/AT6 PCR product	This study
pDDA0749-2	pCR2.1-AT56 containing EZ::TN<KAN-2> transposon within *bimA*	This study
pCR2.1-AT78	pCR2.1-TOPO containing AT7/AT8 PCR product	This study
pDDA0747-2	pCR2.1-AT78 containing EZ::TN<KAN-2> transposon within *bimE*	This study
pCR2.1-AT910	pCR2.1-TOPO containing AT9/AT10 PCR product	This study

Table 5. *cont.*

Strain or plasmid	Relevant characteristics[b]	Reference
pDDA0745	pCR2.1-AT910 containing EZ::TN<KAN-2> transposon within *virA*	This study
pGRV2-ΔA0739	pGRV2 containing EcoRI insert from pCR2.1-ΔA0739	This study
pGRV2-ΔA0740	pGRV2 containing EcoRI insert from pCR2.1-ΔA0740	This study
pGRV2-ΔA0742	pGRV2 containing EcoRI insert from pUCP30T-Δ42	This study
pGRV2-ΔA0743	pGRV2 containing EcoRI insert from pCR2.1-ΔA0743	This study
pA0745	pGRV2 containing EcoRI insert from pDDA0745, Km^R	This study
pGRV2-ΔA0746	pGRV2 containing XbaI–PinAI insert from pCRT7/CT-AT1112ΔE	This study
pA0747-2	pGRV2 containing EcoRI insert from pDDA0747-2, Km^R	This study
pA0749-2	pGRV2 containing EcoRI insert from pDDA0749-2, Km^R	This study
pGRV2-ΔA0750	pGRV2 containing XbaI–PinAI insert from pCRT7/CT-AT34ΔE	This study
pA0751-3	pGRV2 containing EcoRI insert from pDDA0751-3, Km^R	This study

a. The USAMRIID Institutional Biosafety Committee does not believe that introduction of the gentamicin resistance marker into *B. mallei* compromises the ability to treat glanders because gentamicin is not indicated for treatment of this disease (Srinivasan *et al*., 2001; Bossi *et al*., 2004; Currie, 2005). However, because this work may not be allowed under the US Code of Federal Regulations (CFR), title 42, part 73 (42 CFR 73) and 9 CFR 121, we have asked the Division of Select Agents and Toxins, CDC, for clarification and are awaiting a response. However, we have destroyed strains harbouring pSCrhaB2 derivatives because there is a possibility that trimethoprim-sulfamethoxazole could be used for glanders eradication therapy (Bossi *et al*., 2004; Currie, 2005).
b. Cm, chloramphenicol; R, resistant; S, sensitive.

23344 (pSCRhaB2). Four separate expression profiling experiments were performed and the combined results of two representative experiments from two different investigators are shown in Table 1. With a few exceptions, only genes induced twofold in all four experiments are shown. RNA was isolated from ATCC 23344 (pSCrhaB2-*BMAA1517*) at mid-log phase in the presence and absence of L-rhamnose and processed as described above. Three separate expression profiling experiments were performed and the average of all experiments is shown in Table 2.

Immunogold electron microscopy

Bacteria were grown in LBG broth to mid-log phase and placed on a Formvar-coated nickel grid (400 mesh) for 2 min. The grids were blocked for 30 min with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.3 (PBS), and a 1:50 dilution of the anticapsule monoclonal antibody 4VA5 (Nelson *et al*., 2004) was added for 30 min The grids were washed three times with PBS/BSA, and a 1:50 dilution of a goat anti-mouse IgG gold conjugate (5 nm; Sigma) was

Table 6. Oligodeoxyribonucleotide primers used in this study.

Primer name	Primer sequence (5′ to 3′)	Target
BMAA0742-up	ATGCTGGCCGGAATATATC	*hcp1*
BMAA0742-dn	GCCATTCGTCCAGTTTGC	*hcp1*
39-up	GAGCTGTATCTGTTCGGCTG	*tssE*
39-dn	AGGGGTTGAGCTGTTCGATC	*tssE*
40-up	AAAAGCTCGACGAAGGCCTG	*tssD*
40-dn2	TGTTCGTCGCGTTCCTCGAC	*tssD*
42-up	CAAGCTCGAAAAGCGCTAGG	*hcp1*
42-dn	AGGCCTTCGTCGAGCTTTTG	*hcp1*
IGR1	AGGTCTCAGAGGTCGGCGAA	*tssB*
43-dn	ACGAAGATGTACGGCAGTTG	*tssB*
AT1	ATCATTCGAGCACGATCGTG	*bimB*
AT2	CGCTCGATGTATTTGTCCAG	*bimB*
AT3	CTTAGTCGAACGTAACGAAG	*bimC*
AT4	GCGTTAAACGCCGTACTTTC	*bimC*
AT5	TTCGATCGATTCCTGCTATC	*bimA*
AT6	TTGTCGATGTGACGCGTTAC	*bimA*
AT7	GTCACATCGACAAACGCATG	*bimE*
AT8	AAAGAGGTGGAGACGCTATG	*bimE*
AT9	ATAGCGTCTCCACCTCTTTC	*virA*
AT10	TGGATGAACGACCGCGAATG	*virA*
AT11	AATGACGTTCGACGAGATCG	*virG*
AT12	TCTTCGAGCCGTTCTATTCG	*virG*
AT14	TCATCCCCATAGCGTCTCCAC	*virAG*
AT15	CGACCTCTGAGACCTATGCAC	*virAG*
virAG_3	GCGAGCGCTTGGACATCGATCG	*virAG*
virAG_5	GTTCTACGCGAATCATCTGCTGTC	*virAG*
GFP-up	CTTTGTTAGCAGCCGGATCC	*gfp*
GFP-dn	CCCCTCTAGA AATAATTTTG	*gfp*
S1520F	CGTTCCGCACGGATTCTC	*BMAA1517*
S1520R	GTCGGAATCGACATTCATCGT	*BMAA1517*

added for 30 min The grids were washed three times with PBS/BSA and examined on a JEM 1011 transmission electron microscope (JOEL USA).

Host cell infection and actin tail formation

J774.2 murine macrophage-like cells were infected with either *B. mallei* SR1 (pBHR4-GFP), DDA0746 (pBHR4-GFP), DDA0747 (pBHR4-GFP), DDA0749 (pBHR4-GFP), DDA0750 (pBHR4-GFP) or DDA0751 (pBHR4-GFP) at a multiplicity of infection of 10 bacteria per macrophage in six-well plates. Cells were centrifuged for 5 min at 1500 r.p.m. and incubated for 1 h at 37°C with 5% CO_2. Cells were washed three times with PBS and overlaid with medium containing 100 μg ml^{-1} of streptomycin to prevent the growth of extracellular bacteria. At 6 h post infection, cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Cells were permeabilized with 1% Triton X-100, and filamentous actin was stained red by overnight incubation at 4°C with Alexa Fluor^{568}-phalloidin diluted to 5 U ml^{-1} in PBS (Molecular Probes, Eugene, OR). The nucleus was stained blue with 4′,6-diamidino-2-phenylindole (DAPI). A Nikon Eclipse TE2000-S inverted microscope equipped with a Spot-RT digital camera (Image Systems, Columbia, MD) was used to visualize J774.2 cells infected with *B. mallei* strains.

Animal studies

Three groups of female Syrian hamsters (five per group) were infected by the intraperitoneal route with a range of 10^1–10^4 cfu for each strain of *B. mallei* examined. Mortality was recorded daily for 14 days and on day 15, the surviving animals from each group were euthanized.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Preparation of supernatant proteins and trypsin digestion

Strains were grown for 24 h in 125 ml disposable Erlenmeyer flasks containing 25 ml of LBG and appropriate antibiotics. When appropriate, L-rhamnose was added to the culture medium. Cultures were transferred to centrifuge bottles and cell pellets were obtained after centrifugation at 4500 r.p.m. for 15 min at 10°C. Supernatants were filter sterilized through 0.45 mm Millex HV filters (Millipore) and protein concentrations were determined (~50 μg ml^{-1}) using the BCA Protein Assay Kit (Pierce). Supernatant proteins were precipitated by adding 8 ml of ice-cold acetone (Sigma) and 1 ml of 100% trichloroacetic acid (Sigma) to 1 ml of supernatant solution. After an overnight incubation, the solutions were centrifuged at 18 000 × g for 15 min at 4°C to obtain protein pellets. The protein pellets were washed with 1 ml of ice-cold acetone, centrifuged at 18 000 × g for 10 min at 4°C, and allowed to dry at room temperature for 30 min.

The precipitated supernatant proteins were dissolved in 8 M urea/0.1 mM ammonium bicarbonate (pH 8.0) (Sigma) and disulphide bonds were reduced by adding 0.045 M dithiothreitol (Sigma) for 15 min at 60°C. After cooling to room temperature, 0.1 M iodoacetamide (Sigma) was added and samples were incubated at 60°C for 15 min. The samples were allowed to cool to room temperature and diluted to a final volume of 200 μl with 0.025 M ammonium bicarbonate (pH 8.0). One microgram of sequencing grade porcine trypsin (Promega) was added to each protein solution and incubated at 37°C for 16 h. Trypsin digestion was stopped by the addition of formic acid and the samples were stored at –20°C.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Tryptic peptide data were acquired using a Finnigan LTQ linear ion trap mass spectrometer equipped with a nanoelectrospray source and a Finnigan Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA). Eight microliters of each tryptic digest were injected onto a New Objective (Woburn, MA) capillary LC column (10 cm × 75 μm) packed with Aquasil C18 (5 μm particle size, 100 angstrom pore size). Peptides were eluted using a flow rate of 500 nl min^{-1} with flow splitting and the following linear gradient: 0–80% B in 60 min. Solvents A and B consisted of 0.1% formic acid (Sigma) and 90% acetonitrile (Sigma) in 0.1% formic acid respectively. The electrospray voltage was 2.0 kV. The mass spectrometer was operated using data-dependent MS/MS acquisition.

Protein identification was performed using Bioworks 3.2 software (Thermo Electron Corporation, San Jose, CA) and the NCBInr database (13Oct06). Identified peptides were further evaluated using a *B. mallei* taxonomy filter and charge state versus cross-correlation scores (XCorr). The XCorr criteria for positive identification of tryptic peptides were values > 1.9 for singly charged ions, values > 2.2 for doubly charged ions, and values > 2.9 for triply charged peptides.

Cloning, expression and purification of V5-tagged Hcp1

The *B. mallei* ATCC 23344 *hcp1* gene was PCR-amplified with BMAA0742-up and BMAA0742-dn (Table 6) and the 507 bp product was cloned into pCRT7/CT-TOPO (Invitrogen). The resulting plasmid, pCRT7/CT-A0742, contained *hcp1* fused to a C-terminal V5 epitope and a polyhistidine (6XHis) tag. The plasmid was transformed into *E. coli* Origami (DE3) and the strain was grown in a 125 ml disposable Erlenmeyer flask containing 50 ml of LB with Cb, Tc and Km. After 2 h of growth, IPTG was added and the culture was incubated an additional 4 h. The recombinant HcpV5 protein was isolated using the ProBond Purification System (Invitrogen), following the supplied protocol for purification under native conditions. Immobilized metal ion affinity chromatography (IMAC) was carried out in batch mode using ProBond resin and the 200 mM imidazole was step was performed three times instead of once. The recombinant HcpV5 protein

was eluted with 500 mM imidazole, dialysed in 1 × PBS (Teknova) overnight at 4°C, and concentrated using a Centricon Plus-20 centrifugal filter device (Millipore). The BCA Protein Assay Kit was used to determine protein concentration and purity was assessed by SDS-PAGE on 10–20% Tris Glycine Gels (Invitrogen) using 1 × TrisGly SDS Running Buffer (Invitrogen). Protein gels were stained using the Colloidal Blue Staining Kit (Invitrogen).

Immunoblotting with anti-V5-HRP and glanders sera

Wild-type and T6S mutant strains were grown for 8 h in 14 ml snap-cap tubes containing 3 ml of LBG for HcpV5 immunoblotting experiments. One millilitre volumes were transferred to microcentrifuge tubes and cell pellets were obtained after centrifugation at 14 000 r.p.m. for 2 min at 25°C. Cell pellets were washed once with 1 × PBS, resuspended in 1 ml of 1X Tris Glycine SDS Sample Buffer (Invitrogen), boiled for 10 min, and stored at 4°C. Three microliters of sample was loaded onto a 10–20% Tris Glycine Gel and proteins were separated by electrophoresis using 1 × TrisGly SDS Running Buffer. Supernatants were filter sterilized through 0.45 mm Millex HV filters (Millipore) and 100 μl was precipitated at −20°C for 30 min using 10% trichloroacetic acid. Protein pellets were collected by centrifugation (14 000 r.p.m. for 10 min) and washed once with cold acetone. The protein pellets were resuspended in 1 × Tris Glycine SDS Sample Buffer and processed as described above. Proteins were transferred to Invitrolon PVDF (Invitrogen) using a XCell SureLock apparatus (Invitrogen). The membranes were blocked with 3.5% skim milk powder (EMD Chemicals), 0.1% Tween 20 (Sigma), incubated with a 1:20 000 dilution of anti-V5-HRP antibody (Invitrogen), and developed with TMB Membrane Peroxidase Substrate (KPL).

For immunoblot analysis of IMAC-purified HcpV5, ~500 ng of recombinant protein was processed as described above except that anti-V5-HRP antibody was used at a 1:30 000 dilution. Human glanders antiserum was obtained from a glanders patient approximately 3 months after an accidental *B. mallei* laboratory-acquired infection (Srinivasan *et al.*, 2001). Pre-infection serum was obtained from the same individual approximately 3 months before the glanders infection. The human serum samples were used at a 1:10 000 dilution. The secondary antibody, peroxidase-labelled goat anti-human IgG (H + l) (KPL), was used at a 1:5000 dilution. Horse glanders antiserum, a kind gift from Reena Zachariah (CVRL, Dubai, UAE), was obtained from a horse involved in a recent outbreak of glanders in Al Ain, United Arab Emirates (Scholz *et al.*, 2006) and was used at a 1:10 000 dilution. Normal horse serum was obtained from a horse in UAE that was not involved in the glanders outbreak. Peroxidase-labelled goat anti-horse IgG (H + l) (KPL) was used as the secondary antibody at a 1:5000 dilution. Mouse glanders antisera were obtained 77 days post infection from a group of five BALB/c mice that were chronically infected with a sublethal aerosol dose of *B. mallei* NCTC 10229. Control mouse sera were obtained from a group of five uninfected BALB/c mice. Pooled glanders and control mouse sera ($n = 5$) were used at a 1:1000 dilution and the secondary antibody, peroxidase labelled goat anti-mouse IgG (H + l) (KPL), was used at a 1:5000 dilution.

Nucleotide sequence data

Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the Accession number TPA: BK006128.

Acknowledgements

We thank Anthony Bassett and Kathy A. Kuehl for technical assistance and Henry S. Heine for chronic glanders mouse sera samples. We also thank Mitali Sarkar-Tyson and Reena Zachariah for monoclonal antibody 4VA5 and glanders horse serum respectively. The research described herein was sponsored by the Medical Biological Defense Research Program, US Army Medical Research and Materiel Command, project 06-4-2P-004 (D.D.), NIAID Interagency Agreement Y1-AI-5004-01 (D.D.) and National Institutes of Health Grant 1-R21-AI069081 (M.A.S.). Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army in accordance with AR 70-31.

References

Benz, I., and Schmidt, M.A. (2001) Glycosylation with heptose residues mediated by the *aah* gene product is essential for adherence of the AIDA-I adhesin. *Mol Microbiol* **40:** 1403–1413.

Bladergroen, M.R., Badelt, K., and Spaink, H.P. (2003) Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. *Mol Plant Microbe Interact* **16:** 53–64.

Bossi, P., Tegnell, A., Baka, A., van Loock, F., Hendriks, J., Werner, A., *et al.* (2004) Bichat guidelines for the clinical management of glanders and melioidosis and bioterrorism-related glanders and melioidosis. *Euro Surveill* **9:** 1–6.

Brundage, R.A., Smith, G.A., Camilli, A., Theriot, J.A., and Portnoy, D.A. (1993) Expression and phosphorylation of the *Listeria monocytogenes* ActA protein in mammalian cells. *Proc Natl Acad Sci USA* **90:** 11890–11894.

Cardona, S.T., and Valvano, M.A. (2005) An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. *Plasmid* **54:** 219–228.

Carlsson, F., and Brown, E.J. (2006) Actin-based motility of intracellular bacteria, and polarized surface distribution of the bacterial effector molecules. *J Cell Physiol* **209:** 288–296.

Chain, P.S., Denef, V.J., Konstantinidis, K.T., Vergez, L.M., Agullo, L., Reyes, V.L., *et al.* (2006) *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. *Proc Natl Acad Sci USA* **103:** 15280–15287.

Currie, B.J. (2005) *Burkholderia pseudomallei* and *Burkholderia mallei*: melioidosis and glanders. In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, Vol. 2. Mandell, G.L., Bennett, J.E., and Dolin, R. (eds). New York: Churchill Livingstone, pp. 2622–2632.

Das, S., and Chaudhuri, K. (2003) Identification of a unique IAHP (IcmF associated homologous proteins) cluster in

Vibrio cholerae and other proteobacteria through *in silico* analysis. *In Silico Biol* **3:** 287–300.

Dennis, J.J., and Zylstra, G.J. (1998) Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. *Appl Environ Microbiol* **64:** 2710–2715.

DeShazer, D., Brett, P.J., Carlyon, R., and Woods, D.E. (1997) Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. *J Bacteriol* **179:** 2116–2125.

DeShazer, D., Waag, D.M., Fritz, D.L., and Woods, D.E. (2001) Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. *Microb Pathog* **30:** 253–269.

Desvaux, M., Parham, N.J., Scott-Tucker, A., and Henderson, I.R. (2004) The general secretory pathway: a general misnomer? *Trends Microbiol* **12:** 306–309.

Dudley, E.G., Thomson, N.R., Parkhill, J., Morin, N.P., and Nataro, J.P. (2006) Proteomic and microarray characterization of the AggR regulon identifies a *pheU* pathogenicity island in enteroaggregative *Escherichia coli*. *Mol Microbiol* **61:** 1267–1282.

Ferster, L.N., and Kurilov, V. (1982) Characteristics of the infectious process in animals susceptible and resistant to glanders (in Russian). *Arkh Patol* **44:** 24–30.

Finn, R.D., Mistry, J., Schuster-Böckler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., *et al.* (2006) Pfam: clans, web tools and services. *Nucleic Acids Res* **34:** D247–D251.

Folkesson, A., Lofdahl, S., and Normark, S. (2002) The *Salmonella enterica* subspecies I specific centisome 7 genomic island encodes novel protein families present in bacteria living in close contact with eukaryotic cells. *Res Microbiol* **153:** 537–545.

Fritz, D.L., Vogel, P., Brown, D.R., and Waag, D.M. (1999) The hamster model of intraperitoneal *Burkholderia mallei* (glanders). *Vet Pathol* **36:** 276–291.

Harley, V.S., Dance, D.A.B., Drasar, B.S., and Tovey, G. (1998) Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukarotic cells in tissue culture. *Microbios* **96:** 71–93.

Holm, M.M., Vanlerberg, S.L., Sledjeski, D.D., and Lafontaine, E.R. (2003) The Hag protein of *Moraxella catarrhalis* strain O35E is associated with adherence to human lung and middle ear cells. *Infect Immun* **71:** 4977–4984.

Howe, C., and Miller, W.R. (1947) Human glanders: report of six cases. *Ann Intern Med* **26:** 93–115.

Hunter, D.H. (1936) Saints and martyrs. *Lancet* **231:** 1131–1134.

Karlin, S. (2001) Detecting anomalous gene clusters and pathogenicity islands in diverse bacterial genomes. *Trends Microbiol* **9:** 335–343.

Khrapova, N.P., Tikhonov, N.G., and Prokhvatilova, Y.V. (1998) Detection of glycoprotein of *Burkholderia pseudomallei*. *Emerg Infect Dis* **4:** 336–337.

Kim, H.S., Schell, M.A., Yu, Y., Ulrich, R.L., Sarria, S.H., Nierman, W.C., and DeShazer, D. (2005) Bacterial genome adaptation to niches: divergence of the potential virulence genes in three Burkholderia species of different survival strategies. *BMC Genomics* **6:** 174, doi: 10.1186/1471-2164-1186-1174.

Kohler, W. (2006) Killed in action: microbiologists and clinicians as victims of their occupation. Part 4: tick-borne relapsing fever, Malta fever, glanders, SARS. *Int J Med Microbiol* **296:** 1–4.

Lehavi, O., Aizenstien, O., Katz, L.H., and Hourvitz, A. (2002) Glanders-a potential biological warfare against humans and animals. *Harefuah* **141:** 88–91.

Lin, J., Michel, L.O., and Zhang, Q. (2002) CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrob Agents Chemother* **46:** 2124–2131.

Lindenthal, C., and Elsinghorst, E.A. (1999) Identification of a glycoprotein produced by enterotoxigenic *Escherichia coli*. *Infect Immun* **67:** 4084–4091.

Lopez, J., Copps, J., Wilhelmsen, C., Moore, R., Kubay, J., St-Jacques, M., *et al.* (2003) Characterization of experimental equine glanders. *Microbes Infect* **5:** 1125–1131.

McFadyean, J. (1904) Glanders. *J Comp Pathol* **17:** 295–317.

Miller, W.R., Pannell, L., Cravitz, L., Tanner, W.A., and Rosebury, T. (1948) Studies on certain biological characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei*. II. Virulence and infectivity for animals. *J Bacteriol* **55:** 127–135.

Moore, M.M., Fernandez, D.L., and Thune, R.L. (2002) Cloning and characterization of *Edwardsiella ictaluri* proteins expressed and recognized by the channel catfish *Ictalurus punctatus* immune response during infection. *Dis Aquat Organ* **52:** 93–107.

Mougous, J.D., Cuff, M.E., Raunser, S., Shen, A., Zhou, M., Gifford, C.A., *et al.* (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* **312:** 1526–1530.

Nano, F.E., Zhang, N., Cowley, S.C., Klose, K.E., Cheung, K.K., Roberts, M.J., *et al.* (2004) A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *J Bacteriol* **186:** 6430–6436.

Nelson, M., Prior, J.L., Lever, M.S., Jones, H.E., Atkins, T.P., and Titball, R.W. (2004) Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis. *J Med Microbiol* **53:** 1177–1182.

Nierman, W.C., DeShazer, D., Kim, H.S., Tettelin, H., Nelson, K.E., Feldblyum, T., *et al.* (2004) Structural flexibility in the *Burkholderia mallei* genome. *Proc Natl Acad Sci USA* **101:** 14246–14251.

Orr, N., Katz, D.E., Atsmon, J., Radu, P., Yavzori, M., Halperin, T., *et al.* (2005) Community-based safety, immunogenicity, and transmissibility study of the *Shigella sonnei* WRSS1 vaccine in Israeli volunteers. *Infect Immun* **73:** 8027–8032.

Piven, N.N., Smirnova, V.I., Viktorov, D.V., Kovalenko, A.A., Farber, S.M., Yarulin, R.G., and Podzolkova, G.G. (1996) Immunogenicity and heterogeneity of *Pseudomonas pseudomallei* surface antigen 8 (in Russian). *Zh Mikrobiol Epidemiol Immunobiol* **4:** 75–78.

Potvin, E., Lehoux, D.E., Kukavica-Ibrulj, I., Richard, K.L., Sanschagrin, F., Lau, G.W., and Levesque, R.C. (2003) *In vivo* functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets. *Environ Microbiol* **5:** 1294–1308.

Pukatzki, S., Ma, A.T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W.C., *et al.* (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc Natl Acad Sci USA* **103:** 1528–1533.

Rao, P.S., Yamada, Y., Tan, Y.P., and Leung, K.Y. (2004) Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. *Mol Microbiol* **53:** 573–586.

Rhodes, E.R., Tomaras, A.P., McGillivary, G., Connerly, P.L., and Actis, L.A. (2005) Genetic and functional analyses of the *Actinobacillus actinomycetemcomitans* AfeABCD siderophore-independent iron acquisition system. *Infect Immun* **73:** 3758–3763.

Ribot, W.J., and Ulrich, R.L. (2006) The animal pathogen-like type III secretion system is required for the intracellular survival of *Burkholderia mallei* within J774.2 macrophages. *Infect Immun* **74:** 4349–4353.

Rotz, L.D., Khan, A.S., Lillibridge, S.R., Ostroff, S.M., and Hughes, J.M. (2002) Public health assessment of potential biological terrorism agents. *Emerg Infect Dis* **8:** 225–230.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., *et al.* (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34:** 374–378.

Samygin, V.M., Khrapova, N.P., Spiridonov, V.A., and Stepin, A.A. (2001) Antigen 8 biosynthesis during cultivation of *Burkholderia pseudomallei* and *B. mallei* (in Russian). *Zh Mikrobiol Epidemiol Immunobiol* **4:** 50–52.

Schlieker, C., Zentgraf, H., Dersch, P., and Mogk, A. (2005) ClpV, a unique Hsp100/Clp member of pathogenic proteobacteria. *Biol Chem* **386:** 1115–1127.

Scholz, H.C., Joseph, M., Tomaso, H., Al Dahouk, S., Witte, A., Kinne, J., *et al.* (2006) Detection of the reemerging agent *Burkholderia mallei* in a recent outbreak of glanders in the United Arab Emirates by a newly developed *fliP*-based polymerase chain reaction assay. *Diagn Microbiol Infect Dis* **54:** 241–247.

Schweizer, H.P., Klassen, T., and Hoang, T. (1996) Improved methods for gene analysis and expression in *Pseudomonas* spp. In *Molecular Biology of Pseudomonads* Nakazawa, T., Furukawa, K., Haas, D. and Silver, S. (eds). Washington, D.C.: American Society for Microbiology, pp. 229–237.

Simon, R., Priefer, U., and Puhler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: tranposon mutagenesis in gram negative bacteria. *Bio/Technology* **1:** 784–791.

Snel, B., Lehmann, G., Bork, P., and Huynen, M.A. (2000) STRING: a web-server to retrieve and display the repeatedly occurring neighbourhood of a gene. *Nucleic Acids Res* **28:** 3442–3444.

Srinivasan, A., Kraus, C.N., DeShazer, D., Becker, P.M., Dick, J.D., Spacek, L., *et al.* (2001) Glanders in a military microbiologist. *N Engl J Med* **354:** 256–258.

Stevens, J.M., Ulrich, R.L., Taylor, L.A., Wood, M.W., DeShazer, D., Stevens, M.P., and Galyov, E.E. (2005a) Actin-binding proteins from *Burkholderia mallei* and *Burkholderia thailandensis* can functionally compensate for the actin-based motility defect of a *Burkholderia pseudomallei bimA* mutant. *J Bacteriol* **187:** 7857–7862.

Stevens, M.P., Stevens, J.M., Jeng, R.L., Taylor, L.A., Wood, M.W., Hawes, P., *et al.* (2005b) Identification of a bacterial factor required for actin-based motility of *Burkholderia pseudomallei*. *Mol Microbiol* **56:** 40–53.

Stewart, J.C. (1904) Pyaemic glanders in the human subject. *Ann Surg* **40:** 109–113.

Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V., *et al.* (2003) The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* **4:** 41.

Tuanyok, A., Kim, H.S., Nierman, W.C., Yu, Y., Dunbar, J., Moore, R.A., Baker, P., Tom, M., Ling, J.M., and Woods, D.E. (2005) Genome-wide expression analysis of iron regulation in *Burkholderia pseudomallei* and *Burkholderia mallei* using DNA microarrays. *FEMS Microbiol Lett* **252:** 327–335.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98:** 5116–5121.

Ulrich, R.L., and DeShazer, D. (2004) Type III secretion: a virulence factor delivery system essential for the pathogenicity of *Burkholderia mallei*. *Infect Immun* **72:** 1150–1154.

Ulrich, R.L., DeShazer, D., Hines, H.B., and Jeddeloh, J.A. (2004) Quorum sensing: a transcriptional regulatory system involved in the pathogenicity of *Burkholderia mallei*. *Infect Immun* **72:** 6589–6596.

Ulrich, R.L., Amemiya, K., Waag, D.M., Roy, C.J., and DeShazer, D. (2005) Aerogenic vaccination with a *Burkholderia mallei* auxotroph protects against aerosol-initiated glanders in mice. *Vaccine* **23:** 1986–1992.

Voskuhl, G.W., Cornea, P., Bronze, M.S., and Greenfield, R.A. (2003) Other bacterial diseases as a potential consequence of bioterrorism: Q fever, brucellosis, glanders, and melioidosis. *J Okla State Med Assoc* **96:** 214–217.

Waag, D.M., and DeShazer, D. (2004) Glanders: new insights into an old disease. In *Biological Weapons Defense: Infectious Diseases and Counterbioterrorism*. Lindler, L.E., Lebeda, F.J., and Korch, G.W. (eds). Totowa, NJ: Humana Press, pp. 209–237.

Wang, J.S., and Maldonado, M.A. (2006) The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Mol Immunol* **3:** 255–261.

Williams, S.G., Varcoe, L.T., Attridge, S.R., and Manning, P.A. (1996) *Vibrio cholerae* Hcp, a secreted protein coregulated with HlyA. *Infect Immun* **64:** 283–289.

Wilson, K. (1987) Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds). New York: John Wiley & Sons, pp. 2.4.1–2.4.5.

Zheng, J., Tung, S.L., and Leung, K.Y. (2005) Regulation of a type III and a putative secretion system in *Edwardsiella tarda* by EsrC is under the control of a two-component system, EsrA-EsrB. *Infect Immun* **73:** 4127–4137.

Supplementary material

The following supplementary material is available for this article:

Fig. S1. The four *B. mallei* T6S gene clusters are not significantly distinct from the rest of chromosome 2 and were prob-

ably not acquired by recent lateral gene transfer. The *B. mallei* ATCC 23344 chromosome 2 contrasts are displayed for 10 kb (thin dotted line) and 20 kb (solid line) sliding window plots of G+C content (A), G+C content at codon site 3 (B), and codon usage biases (C) (Karlin, 2001). The four T6S gene clusters are identified by gray boxes and are numbered 1 (*BMAA0744-BMAA0727*), 2 (*BMAA0438-BMAA0455*), 3 (*BMAA0396-BMAA0412*), and 4 (*BMAA1897-BMAA1915*). Chromosome 2 is represented as a linear molecule along the horizontal axis from nucleotide 1 to 2,325,379 and 0.5 megabase (Mb) intervals are indicated.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2007.05734.x
(This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.